

1 **Comparison of three blood transfusion guidelines applied to 31 feline donors to**  
2 **minimise the risk of transfusion transmissible infections.**

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19 **Keywords:** blood donor; blood screening; blood safety; cat; Mycoplasma infections;  
20 Retroviridae infections.

21

22 **Abstract**

23 *Objectives* The increased demand for animal blood transfusions creates the need for an  
24 adequate number of donors. At the same time, a high level of blood safety must be  
25 guaranteed and different guidelines (GLs) deal with this topic. The aim of this study  
26 was to evaluate the appropriateness of different GLs in preventing transfusion-  
27 transmissible infections (TTI) in Italian feline blood donors.

28 *Methods* Blood samples were collected from 31 cats enrolled as blood donors by the  
29 owners' voluntary choice over a period of approximately 1 year. Possible risk factors  
30 for TTI were recorded. Based on Italian, European and American GLs, specific TTI,  
31 including haemoplasmas, feline leukemia virus (FeLV), feline immunodeficiency virus  
32 (FIV), *Anaplasma phagocytophilum*, *Ehrlichia* spp., *Bartonella* spp., *Babesia* spp.,  
33 *Theileria* spp., *Cytauxzoon* spp., *Leishmania donovani* sensu lato and feline coronavirus  
34 (FCoV) were screened. Rapid antigen and serological tests and biomolecular  
35 investigations (PCR) were used. Several PCR protocols for haemoplasma and FeLV  
36 DNA were compared to each other.

37 *Results* The presence of at least one recognized risk factor for TTI was reported in all  
38 cats. Results for FIV and FeLV infections were negative using rapid tests, whereas 5  
39 (16.1%) cats were positive for FCoV antibodies. Four (12.9%) cats were PCR positive  
40 for haemoplasma DNA and 1 (3.2%) for FeLV provirus, the latter being positive only  
41 using the most sensitive PCR protocol applied. Other TTI were not detected using PCR.

42 *Conclusion and relevance* Blood safety increases by combining the recommendations of  
43 different GLs. To reduce the risk of TTI, sensitive tests are needed and the choice of the  
44 best protocol is a critical step in improving blood safety. The cost and time of the  
45 screening procedures may be reduced if appropriate tests are selected. To this end, the  
46 GLs should include appropriate recruitment protocols and questionnaire-based risk  
47 profiles to identify suitable donors.

48

49 **Introduction**

50 Recently, the increase of the indications for the veterinary blood transfusion and its  
51 routine use in the veterinary practice caused a rise in the demand for animal donors, At  
52 the same time, a high level of blood safety must be guaranteed to perform this  
53 procedure.

54 Transfusion-transmitted infections (TTI) from apparently healthy and asymptomatic  
55 blood donors represent a well-known threat in blood transfusion, in addition to other  
56 adverse events.<sup>1-4</sup> Therefore, the identification of risk factors and characteristics of “low  
57 risk” blood donors are very important for increasing blood safety. Appropriate  
58 recruitment strategies could also reduce infection risks. In human medicine, the World  
59 Health Organization (WHO) identified regular voluntary non-remunerated donors as  
60 those with the lowest risk for TTI.<sup>5</sup> However, this model cannot be used because  
61 different situations and settings exist in veterinary medicine. In cats, different  
62 prevalences of microorganisms were found based on the source of the feline donors: a  
63 previous study found that laboratory-reared cats and cats housed indoor with no history  
64 of flea or tick infestation were ideal blood donors.<sup>6</sup>

65 Moreover, strategies and procedures ensuring blood safety for TTI change in different  
66 settings.<sup>7</sup> For humans, it is well known that each country has to address specific issues  
67 or constraints that influence the safety of its blood supply, including the incidence and

68 prevalence of TTI, the structure and level of development of blood transfusion services,  
69 and the economic resources available.<sup>5</sup>

70 There are several guidelines (GLs) for testing protocols in veterinary medicine. GLs are  
71 generally developed according to the circumstances and needs of each country. No  
72 single GL can ensure absolute blood safety.<sup>5</sup> Both American and European GLs aim to  
73 standardize veterinary blood transfusion procedures and to guarantee blood safety.<sup>8-10</sup>  
74 Additional GLs have been published in Italy to regulate veterinary blood transfusions in  
75 dogs, cats and horses in this country.<sup>11-12</sup> However, all these GLs differ in some aspects,  
76 such as the microorganism to be screened or the screening methods.<sup>8-10</sup>

77 The aims of this research were i) to evaluate the differences among existing GLs, and ii)  
78 to perform a preliminary investigation on a selected population of feline blood donors  
79 from an Italian blood bank, evaluating how blood safety can be improved using tests  
80 recommended by the different GLs.

81

## 82 **Materials and methods**

### 83 **Comparison of International and National GLs**

84 The main existing GLs for veterinary blood transfusions were compared.<sup>8-12</sup> The  
85 European and American GLs were written by a panel of experts, according to evidence-  
86 based medicine, even if the level of evidence for each topic was not always explicitly

87 declared.<sup>8-10</sup> The Italian GL for animal transfusion was written by a panel of experts  
88 commissioned by the Italian Ministry of Health.<sup>11,12</sup>

89

### 90 **Feline blood donor selection**

91 During the January 2014-January 2015 period, blood samples were collected from cats  
92 enrolled as blood donors at the Blood Bank of the Veterinary Teaching Hospital of the  
93 University of Perugia. Cats were enrolled using the criteria of suitability indicated in the  
94 Italian GL:<sup>11,12</sup> body weight  $\geq 5$  kg, age 2-8 years, docile character, regularly vaccinated  
95 with core vaccines (feline calicivirus, feline herpesvirus, feline parvovirus) and two  
96 non-core vaccines ( *Chlamydia felis*, formerly *Chlamydophila felis*, and feline leukemia  
97 virus, FeLV). Blood donors enrolled in cases of life-threatening emergencies, for which  
98 the national GL recommends fast and restricted screening limited to feline  
99 immunodeficiency virus (FIV), FeLV and *Mycoplasma haemofelis*, were excluded from  
100 the study.

101 The owners of cat blood donors were asked for the following information: complete  
102 history, blood collection date, type of owner (staff or client), identification of the  
103 animal, age, gender, weight, breed, origin (adoption from breed or stray cat or purchase  
104 at a pet shop), type of housing (indoor vs outdoor or mixed), cohabitation with other  
105 cats, ectoparasite treatment and frequency, coat (long or short hair), and laboratory test

106 results. Travel history was not systematically investigated because initially not included  
107 in the information required.

108 For each potential donor, before the collection of blood, a careful physical examination,  
109 was performed, with particular emphasis on the presence of fleas or ticks, and biological  
110 samples were collected by a single operator for clinicopathologic screening, consisting  
111 of a complete blood count, typing of blood group, serum chemistry, urinalysis, and  
112 faecal examination.

113 Blood donation was a voluntary choice by the owners, who signed a written consent  
114 form to authorize blood collection and storage and the use of samples and data for  
115 scientific purposes. Therefore, based on the current regulations of our institutions, the  
116 formal approval of the ethical committee was not needed for this study.

117

### 118 **Sample collection**

119 One ml of blood from each donor was placed into 2 anticoagulated tubes containing  
120 sodium-citrate or EDTA (Ethylene-diamine-tetra-acetic acid) to obtain buffy coat and  
121 plasma, respectively. Three ml of blood were placed in a plain Vacutainer tube (Becton  
122 Dickinson, Milan, Italy) to obtain serum by centrifugation (1000 xg for 10 min). An  
123 aliquot of each sample was stored at -80 °C for further investigations.

124

### 125 **TTI screening**

126 Serum samples were screened by rapid test using an ELISA for FeLV antigen and FIV  
127 antibodies (SNAP FIV/FeLV Combo test, IDEXX Laboratories) and an  
128 immunochromatographic method for feline coronavirus (FCoV) antibodies (FASTest  
129 FIP, MegaCor Diagnostik).

130 For each cat a Romanowsky-stained blood smear was microscopically examined to  
131 assess the presence of morphologically detectable microorganisms, with attention given  
132 in particular to haemoplasmas.

133 DNA and RNA were extracted from 200  $\mu$ L of both buffy coat and whole blood  
134 samples according to validated protocols (Supplementary Table),<sup>13-25</sup> using a  
135 commercial kit for viral RNA and DNA and bacterial DNA (QIAamp cador Pathogen  
136 Mini Kit, Qiagen, Milan, Italy), in accordance with the manufacturer's instructions. The  
137 concentration and purity of the extracted nucleic acids were quantified using a  
138 NanoDrop® spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan,  
139 Italy).

140 Previously published PCR assays, used for the diagnostic activities in the laboratory that  
141 performed the tests, were applied to detect the infectious agents (Supplementary  
142 Table).<sup>13-25</sup> Several published PCR protocols were performed in the case of  
143 haemoplasmas<sup>13-15</sup> and FeLV<sup>18-20</sup> to compare the sensitivity of the assays. In this case,  
144 10-fold dilutions of positive samples were used.



145 A PCR targeting the 18S ribosomal RNA gene was used as internal control to rule out  
146 possible PCR inhibitors in the samples.

147 With regards to FeLV, the PCR product of the expected size was purified with an  
148 extraction kit (Qiaquick PCR purification kit, Qiagen) and directly sequenced on both  
149 strands with the specific primers previously described,<sup>20</sup> using a DNA analyzer (ABI  
150 3730, Applied Biosystems) capillary sequencer (Bio-Fab Research srl). The sequences  
151 were assembled and aligned using BioEdit software<sup>26</sup> and sequence similarities were  
152 assessed by comparison with the sequences deposited in GenBank using the BLAST  
153 software.<sup>27</sup>

154

### 155 **Statistical analysis**

156 Chi-square, with Yates's correction, and Fisher's exact test were used to compare the  
157 proportions of positive and negative samples, stratified for the data of the animals at the  
158 time of the visit (staff- or client-owner, age, gender, breed, origin, type of housing,  
159 cohabitation with other cats, ectoparasite treatment and frequency, kind of coat), as  
160 most appropriate. Age was analysed by grouping the animals into age categories testing  
161 a cut-off  $\leq 3$  years. A P value  $<0.05$  was considered statistically significant. EpiInfo<sup>28</sup>  
162 and OpenEpi<sup>29</sup> were used for analysis.

163

### 164 **Results**

## 165 **Comparison of International and National GLs**

166 Different aspects concerning criteria of suitability, selection and TTI screening for  
167 blood donors are reported in the GLs.<sup>8-12</sup> However, all GLs consider FIV, FeLV, and  
168 *Mycoplasma haemofelis* as the minimum essential level of screening (Tables 1 and 2).  
169 Based on the epidemiological situation of the individual country<sup>14,30-37</sup> and as suggested  
170 by all GLs, Italian GLs were partially integrated with European and American GLs for  
171 increasing blood safety. Accordingly, further TTI were investigated: *Candidatus*  
172 *Mycoplasma haemominutum*, *Candidatus Mycoplasma turicensis*, *Anaplasmataceae*  
173 family (*Anaplasma phagocytophilum* and *Ehrlichia* spp.), *Bartonella* spp., *Babesia* spp.,  
174 *Theileria* spp., *Cytauxzoon* spp., *Leishmania donovani* sensu lato, and FCoV. In  
175 addition, biomolecular methods were added to the procedures recommended by the  
176 Italian GL.<sup>12</sup>

177

## 178 **Feline blood donor profiles**

179 Thirty-one cats from 18 different owners were included in the study. Six (19.4%) were  
180 female (3 neutered) and 25 (80.6%) male (15 neutered). The mean age was 4 years  
181 (median 5, range 2-8) and the mean weight 6.23 kg (median 6, range 5-9). Twenty-three  
182 (74.2%) were domestic shorthair, whereas 8 (25.8%) were Maine Coon. Twenty cats  
183 (64.5%) were client-owned and 11 (35.5%) staff-owned, including students of the  
184 veterinary medicine course. Based on the answers received, 22 (71%) cats were adopted

185 stray kittens, whereas 9 (29%) were purebred cats. Thirteen (44.8%) cats lived indoors,  
186 whereas 16 (55.2%) had access to the outdoors. Twenty-six (83.8%) lived with other  
187 cats. Only 2 owners performed regular ectoparasite treatments, whereas 25 others  
188 (89.3%) administered the treatments mainly in warm periods, and 1 never administered  
189 ectoparasite treatments. Thirteen cats (46.4%) were long-haired, and 15 (53.6%) short-  
190 haired. In three cases the recording of the answers missed to collect information on the  
191 kind of coat and treatment, and in two cases the type of housing.

192 At least 5 owners travelled with their cats in different areas of southern-central Italy.

193 No cats had recent illnesses. Physical examination and clinicopathologic screenings  
194 were unremarkable, with all the parameters within the standard reference intervals, and  
195 cats were considered eligible for blood donation.

196

#### 197 **TTI screening**

198 All cats were negative using FIV and FeLV rapid tests, whereas 5 (16.1%) were  
199 positive for FCoV antibodies.

200 Blood smears did not reveal morphologically detectable microorganisms.

201 Cats were negative using PCR for *Anaplasmataceae* (*Anaplasma* spp. and *Ehrlichia*  
202 spp.), *Bartonella* spp., *Babesia* spp., *Theileria* spp., *Cytauxzoon* spp., *Leishmania*  
203 *donovani* sensu lato and FCoV, whereas haemoplasma DNA was detected in 4 cats  
204 (12.9%) using all the PCR protocols targeting the 16S rRNA gene.<sup>13-15</sup> However, the

205 nested PCR<sup>14</sup> was found to be 10-fold more sensitive than the other PCR protocol  
206 used.<sup>13,15</sup> Further specific tests<sup>16,17</sup> identified three cats as infected with *Candidatus*  
207 *Mycoplasma haemominutum* and one with *Mycoplasma haemofelis*. The latter cat was  
208 also PCR positive for FeLV provirus using the specific nested PCR protocol,<sup>19</sup> but not  
209 with the others protocols used to detect FeLV DNA.<sup>18,20</sup> The specific nested protocol<sup>19</sup>  
210 was at least 10-fold more sensitive than the others.<sup>18,20</sup> Sequencing of the positive FeLV  
211 PCR product shared 98%-100% identity with the homologous FeLV region LTR  
212 sequences reported in GenBank (accession nos. [AY374218](#), [L25630](#), [M18248](#),  
213 [KP728112](#)).

214

### 215 **Statistical analysis**

216 No statistical association with positivity to the screened microorganisms and the  
217 characteristics of the cats was found.

218

### 219 **Discussion**

220 In this study, Italian GLs were applied to 31 feline candidate blood donors enrolled over  
221 a 1-year period. According to these GLs, these cats were fully eligible as blood donors.  
222 However, based on donor selection criteria or recommendations of other GLs, blood  
223 safety could not be completely guaranteed. Indeed, the enrolled cats, although clinically

224 healthy, had risk factors for harbouring TTI and resulted also positive for some  
225 microorganisms included in the other GLs.

226 All 31 cats were negative using rapid tests for FIV and FeLV. One cat was positive for  
227 FeLV provirus, but only when using the most sensitive PCR protocol. This cat should  
228 be excluded from the donation program, considering that FeLV provirus carriers testing  
229 negative for the p27 antigen may transmit FeLV infection to a naïve recipient via blood  
230 transfusion.<sup>38</sup> These results show that the Italian GL would have missed this infected cat  
231 because, as opposed to the other GLs,<sup>9,10,39</sup> PCR is not even recommended for detecting  
232 proviral FeLV. Furthermore, the results also raised the issue that the same animal may  
233 be classified eligible or ineligible as a blood donor based on the different PCR assay  
234 that is applied.

235 Blood smears negative for haemoplasmas cannot exclude infection. The sensitivity of  
236 microscopic analysis is too low to detect haemoplasmas in chronically asymptomatic  
237 cats and does not differentiate the *Mycoplasma* species.<sup>40,41</sup> Using sensitive PCR  
238 protocols, as suggested by European and American GLs and previous studies,<sup>2,3,8,10</sup>  
239 12.9% of cats were positive, with a prevalence comparable with that of previous studies  
240 on blood donors.<sup>37,40,42</sup> This confirms the endemicity of haemoplasma in clinically  
241 healthy cats and the high probability of finding a positive cat even in a limited number  
242 of samples.<sup>6,40</sup> All the PCR protocols<sup>13-15</sup> identified the positive cats, but the nested  
243 PCR<sup>14</sup> was 10-fold more sensitive than the other protocols. This protocol should be

244 preferred for its higher sensitivity, since fluctuations in the number of circulating  
245 haemoplasmas may lead to false negative results using less sensitive protocols.<sup>41</sup> For the  
246 same reason, TTI screening should always be performed on the same blood collected for  
247 donation: negative results for blood samples collected even few days before the  
248 donation cannot rule out the presence of hemoplasmas in subsequent blood samples  
249 used for transfusion because of the fluctuation in bacteremia. Moreover, considering  
250 that the highest risk of haemoplasma transmission by blood was found within one week  
251 from its collection<sup>2</sup> and that the risk in feline blood donation was recently defined non-  
252 negligible,<sup>37</sup> sensitive PCR tests should be recommended.

253 *Mycoplasma haemofelis*, the only species included in the Italian and updated American  
254 GLs, was detected in only one cat, whereas three cats were positive for *Candidatus*  
255 *Mycoplasma haemominutum*. The Italian GL recommends temporarily excluding cats  
256 infected by *Mycoplasma haemofelis* from the donation program until PCR results are  
257 negative, whereas American GLs exclude them permanently, assuming that these cats  
258 may be chronic carriers of *Mycoplasma*.<sup>41,43</sup> The identification of haemoplasma species  
259 is considered optional by the American GL.<sup>9</sup> However, four haemoplasma species are  
260 recognized in cats, and their pathogenic significance is debated:<sup>40,41,44</sup> hence, the  
261 identification of *Mycoplasma* species, although time-consuming, may be recommended.  
262 Although not currently recommended by all GLs, the screening of FCoV antibodies  
263 using rapid tests, as is required by the Italian GL, showed a low prevalence of infection

264 (16.1%) compared to previous studies in Italian populations.<sup>35,45</sup> Furthermore, a specific  
265 nested PCR assay demonstrated that blood samples were negative for FCoV. However,  
266 the European GL<sup>10</sup> and a recent study<sup>46</sup> advise that seropositive cats should be excluded  
267 from the donor program because it is possible that passively transferred anti-FCoV  
268 antibodies could endanger the recipient, if infected, even if no evident risk of  
269 transmission of FCoV via blood has been demonstrated:<sup>46,47,48,49</sup> and no reports of  
270 transmission following blood transfusion have been described until now. On the other  
271 hand, there is no evidence that seropositive cats will develop FIP.<sup>9,10</sup> With the shortage  
272 of donor cats already available, the exclusion of FCoV seropositive cats is likely  
273 unnecessary and could cause a relevant reduction in the number of blood donor cats.  
274 Moreover, considering that transitory viremia is possible even in seronegative cats,<sup>45,50</sup>  
275 FCoV RNA screening of blood collected for donation could be appropriate.

276 The comparison of analytical sensitivities of the different protocols was limited to  
277 haemoplasmas and FeLV PCR assays. However, this approach would have been  
278 appropriate also for the other tests. Therefore validated and common protocols could be  
279 described and advised by GLs in order to guarantee the same level of accuracy in  
280 detecting TTI blood donors everywhere, as is done for notifiable diseases.<sup>51</sup> As in  
281 people, however, test accuracy may be time-consuming and expensive. Otherwise, a  
282 lower level of risk assessment should be accepted by the owner of the recipient by

283 written consent, such as in the case of emergency transfusions, as is currently indicated  
284 by all GLs.<sup>9,10,12</sup>

285 Accordingly, additional appropriate strategies for selecting “low risk donors” should be  
286 applied to reduce the cost and time of screenings and to guarantee a high level of blood  
287 safety. For example, the use of a questionnaire to identify suitable donors could be an  
288 inexpensive and useful tool. As is already the practice in human medicine, recently both  
289 American and European GLs provided different questionnaires to determine the risk  
290 profile.<sup>9,10</sup> However, even if the questionnaire was not available at the time of enrolment  
291 in the study, the owners in the current study were asked specific questions and the  
292 profiles of the donor candidates showed that they had risk factors for harbouring TTI.

293 All 31 Italian donor cats had at least one TTI-risk characteristic or behavior, such as  
294 access to the outdoors, for which American GL specifically recommends repeated  
295 testing,<sup>9</sup> or the irregular ectoparasite treatment performed by 89.3% of the owners. All  
296 GLs advise regular ectoparasite treatments, but the frequency of treatments is not  
297 specified. Monthly ectoparasite prophylaxis was suggested by other studies.<sup>3,7</sup> The  
298 owners should be encouraged to carry out ectoparasite prophylaxis correctly,  
299 considering the relevance of this practice in the control of a wide range of vector-borne  
300 diseases. Although travel history was not systematically investigated, at least five  
301 owners travelled with their cats in different areas of southern-central Italy, that are at  
302 risk of some infectious diseases and therefore should be considered at high risk for



303 harbouring TTI.<sup>33,34</sup> Unfortunately, the small number of cats in this study limited the  
304 statistical power of this study. A higher number of animals could improve the  
305 identification of specific risk factors for TTI in Italy.

306 The possible exclusion of cats with a TTI risk profile from donor programmes contrasts  
307 with the difficulty of finding a sufficient number of adequate donors, as is confirmed by  
308 the enrolment of only 31 cats in more than 1 year. This makes the rejection of candidate  
309 donors very difficult, even if they have a TTI risk profile. Thus the application of a wide  
310 range of sensitive diagnostic tests should be proposed to verify the actual infectious  
311 status and to guarantee a sufficient number of adequate donors. American and European  
312 GLs recommend that donors with a high risk profile should undergo frequent or  
313 extensive TTI screening.<sup>9,10</sup>

314 Another possible action for increasing blood safety that is currently not recommended  
315 by GLs is a recruitment strategy that can identify populations of low TTI-risk donors.  
316 No general recruitment criteria are reported in veterinary medicine, and appropriate  
317 surveys should be taken to identify the best strategy. Laboratory-reared cats could be  
318 considered the ideal donors,<sup>6</sup> being negative for all the microorganisms, but enrolment  
319 of these animals is open to possible ethical issues.<sup>10</sup> A recent survey on hospitals with  
320 blood bank or transfusion services found that staff-owned cats are the donors enrolled  
321 most frequently, followed by colonies of feline donors and client-owned cats.<sup>7</sup> These  
322 sources of donors probably reflect different infection risks. Considering the central role

323 of the owner in managing health prophylaxis, determining the lifestyle of cats and  
324 choosing to donate blood, it is possible that informed and motivated owners could be a  
325 key point for safer blood donors that reduces the possible exposure to TTI risk factors.

326

### 327 **Conclusions**

328 Since the GLs recommend different protocols and can classify cats differently as  
329 eligible or ineligible for blood donation, the harmonization of recommendations would  
330 be advisable. This is especially important for the main TTI and for the choice of the  
331 most sensitive screening tests, with possible variations according to local  
332 epidemiological situations. These additional recommendations would improve the  
333 general level of transfusion blood safety. Screening costs and time may be reduced if  
334 appropriate tests are selected. Attention should be put to identify donors that were stray  
335 cats, with irregular or no ectoparasite treatments, travelling, or with outdoor access. The  
336 use of biomolecular method should be recommended, at least in the case of storage in  
337 blood banks, to identify proviral FeLV DNA, considering that rapid test is not  
338 definitively discriminatory, and haemoplasma DNA in blood collected for donation.  
339 Moreover, issues raised by FCoV seropositivity and possible presence of FCoV RNA in  
340 seronegative cats should be further considered.

341 Finally, appropriate recruitment protocols currently not considered in GLs, educational  
342 courses for owners, the possibility of establishing permanent groups of safe blood

343 donors and questionnaire-based risk profiles could improve the identification of suitable  
344 donors with low risk of harbouring TTI, reducing the necessity to perform extensive  
345 screening.

346

### 347 **Acknowledgements**

348 The authors thank Mr. Carlo Sanesi for his skillful technical assistance.

349

### 350 **Conflict of interest**

351 The authors do not have any potential conflicts of interest to declare.

352

### 353 **Funding**

354 The authors received no specific grants from any public, commercial or non-profit  
355 funding agencies for the preparation of this article.

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