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1 **P22 protein complex in the serodiagnosis of animal tuberculosis: Antigenic stability and**

2 **cross-reactivity with *Corynebacterium pseudotuberculosis* infection**

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39 **Abstract**

40 The P22 ELISA was recently developed for the serodiagnosis of animal tuberculosis. Herein, the
41 stability of the P22 antigen in different presentations and storage conditions, and the cross-
42 reactivity with *Corynebacterium pseudotuberculosis* infection in small ruminants were
43 evaluated. For the stability assay, serum samples from cows, sheep, goats, alpacas, badgers, and
44 wild boar were used in the P22 ELISA. The cross-reactivity analysis used sera from sheep and
45 goats with caseous lymphadenitis (CLA). Differences in the immune recognition of P22 were
46 found when the antigen was stored at 40 °C, but without altering the negative or positive status
47 of each sample. P22 ELISA presented 5.71% cross-reactivity when CLA-positive sheep were
48 evaluated, but no cross-reaction was observed among CLA-positive goat serum samples. This
49 study showed that the P22 protein complex is stable under different formulations and
50 temperatures, and that the assay presents a low cross-reactivity with CLA.

51

52 **Keywords:** *Mycobacterium tuberculosis* complex; caseous lymphadenitis; immunodiagnosis;
53 wildlife; livestock; zoonosis.

54

55 ## 1. Introduction

56 Animal tuberculosis (TB) is a zoonotic infectious disease caused by members of the 57 *Mycobacterium tuberculosis* complex (MTC) [1], which control is relevant for livestock 58 industry, public health and wildlife conservation. TB has been reported in many domestic and 59 wild animals, and some of which can act as reservoirs [2,3].

60 Different diagnostic techniques and target antigens have been reported for the 61 serodiagnosis of TB in animal species. The P22 protein complex has been used as an antigen in 62 enzyme-linked immunosorbent assays (ELISAs), presenting significant levels of sensitivity and 63 specificity, and no significant cross-reactivity with other mycobacteria (e.g., *Mycobacterium* 64 *avium* subspecies *paratuberculosis*) [4-9]. Several factors affect the diagnostic performance of 65 ELISAs, including antigen conformation and stability, which influence the accessibility of 66 epitopes to antibodies [10]. Since the P22 ELISA test has been designed for use under both 67 laboratory and field conditions, such as in farms or in natural environments, it is important to 68 evaluate the antigen stability with the objective to determine if this protein complex can maintain 69 its immune recognition under stress situations.

70 MTC is closely related to *Corynebacterium pseudotuberculosis*, the etiologic agent of the 71 caseous lymphadenitis disease of sheep and goats, and animals from a same geographical area 72 can be subjected to the infection by both bacteria [11]. Given the species similarities, it's 73 possible that diagnostic techniques may present cross-reactivity, although such cross-reaction 74 was not demonstrated in a previous study when using skin tests and interferon-gamma (IFN-ɣ) 75 specific production assays [12]. However, when P22 ELISA was used in a sheep herd where *C.* 76 *pseudotuberculosis* was sporadically isolated, some interference was observed, although it was

77 not significant [6]. Therefore, there was a need to clarify if caseous lymphadenitis may present
78 cross-reactivity in this serological assay.
79 Considering the situation described above, the present work aimed to optimize storage
80 conditions for the P22 protein complex, focusing on its use as an antigen in the serodiagnosis of
81 TB in cattle, goats, sheep, alpacas, badgers, and wild boars. The cross-reactivity with caseous
82 lymphadenitis in small ruminants was also evaluated.
83

84 **2. Material and methods**

85 **2.1 Samples and antigens**

86 The sera used in this study were taken from animals that had their MTC and *C.*
87 *pseudotuberculosis* infectious status confirmed by bacterial isolation (**Table 1**). All the serum
88 samples used herein were aliquoted and kept at -20 °C until use, when the aliquot was thawed at
89 4 °C. No aliquot was used in different times of evaluation.
90 P22 protein complex was obtained and characterized as previously described [13],
91 aliquoted into glass vials as an antigenic solution, or were lyophilized (1 mL, 0.25 mg/mL per
92 vial) and frozen at -20 °C until use. Vials containing P22 in solution or lyophilized were thawed
93 at 4 ºC, aliquoted and stored in a refrigerator or in an incubator at 40 °C for 0, 10, 20 30, 40, 50
94 and 60 days, and then used in the P22 ELISA. For CLA immunodiagnosis, the recombinant
95 phospholipase D (rPLD) was expressed and purified as previously described [14].
96

97 **2.2 ELISA**

98 The P22 antigenic preparations were then used in ELISAs as previously described [4-7],
99 with the objective to analyze the immune recognition of the antigen by serum samples from the

100 different animal species, for each different time point, antigenic formulation and storage
101 temperature. Percent value (E %) was calculated using the following formula:
102 E% = [mean sample optical density / (2 × mean negative control optical density)] × 100%.
103 Samples with a E% ≥ 150 were considered positive for cattle, goat, sheep and badger. An
104 E% ≥ 100 was considered as a cut-off for alpacas and wild boars [4-7].
105 Furthermore, an ELISA using the standard P22 preparation (liquid stored at -20 °C) was
106 conducted to access the cross-reactivity using serum samples from CLA and TB positive sheep
107 and goats. Negative animals had their infectious status confirmed by bacterial isolation and by
108 P22 ELISA, and CLA-positive and -negative goats and sheep were also screened using rPLD as
109 antigen in an indirect ELISA system [14].
110
111 **2.3 Western blot**
112 A Western blot assay was performed to verify the recognition of rPLD and P22 by serum
113 samples from CLA-positive sheep that presented positive results at the P22 ELISA. Briefly, 50
114 µg of rPLD or P22 were subjected to 12% SDS-PAGE and then transferred to nitrocellulose
115 membranes. After blocking with 10% skimmed milk in PBS pH 7.4 for 1h at 37 °C, the
116 membranes were washed two times with PBS-Tween 0.05% (PBS-T) and incubated with the
117 animals' sera and the positive and negative controls for TB and CLA (diluted 1:500 in PBS-T
118 with 1% skimmed milk) for 1h under agitation at 37 °C. After four washes with PBS-T, the
119 membranes were incubated with horseradish peroxidase-conjugated anti-sheep IgG (Sigma-
120 Aldrich) under agitation for 1h. After four washes with PBS-T, the membranes were incubated
121 with 4-chloro-1-naphthol and hydrogen peroxide (Sigma-Aldrich) for 10 min, and the reaction
122 was stopped with ultrapure water.
123

124 **2.4 Statistical analysis**

125 E% was compared between lyophilized P22 and antigenic solution at the same or
126 different temperatures (4 °C; 40 °C). Since the E% within each group followed a normal
127 distribution based on the Shapiro-Wilk test, differences between groups were assessed for
128 significance using ANOVA, followed by Tukey's multiple-comparisons test. Variance within
129 each group was expressed as a percentage, and variances (V%) of a maximum of 20% in the E%
130 were considered as valid and reliable, based on the Spanish Standard ISO/IEC 17025:2017
131 (https://www.iso.org/standard/66912.html).
132
133 **3. Results**
134 **3.1 P22 storage stability**

135 P22 ELISA presented results (E%) that were consistent with TB culture-based diagnosis
136 for all species evaluated herein, independently of P22 storage conditions. All species serum
137 samples met the cut-off criteria previously established [4-7].
138
139 **3.1.1 Domestic species**

140 E% remained stable during the 60 days follow-up period in the ELISAs conducted with
141 serum samples from TB-negative cows, goats and sheep (**Fig. 1**). The E% of lyophilized P22
142 kept at 4 ºC slightly rose over time in the case of TB-negative alpacas (**Fig. 1**). **Table 2** shows
143 that at the ELISAs made with sera from TB-negative cows, V% was significantly smaller ($p <$
144 0.05) with the antigen stored as a solution at 40 °C than with lyophilized antigen stored at the
145 same temperature, and V% was significantly greater with the lyophilized antigen stored at 40 °C
146 than when the same antigen presentation was stored at 4 °C. Similarly, V% in P22 stored as a

147 solution at 4 °C and 40 °C was significantly greater ($p < 0.05$) than both lyophilized antigenic
148 preparations kept at 4 ºC and 40 °C when considering serum samples from negative goats. V%
149 did not differ significantly within different antigen storage conditions in the case of TB-negative
150 sheep, while it differed marginally but not significantly when considering different antigen
151 storage conditions for TB-negative alpacas (**Fig. 1**).

152 A higher V% was observed during the 60-day period in ELISAs made with sera from
153 TB-positive cows, goats, sheep, and alpacas (**Table 3**). For TB-positive cows, V% was
154 significantly smaller when P22 was kept lyophilized than when the same antigenic preparation
155 was stored as a solution at both temperatures ($p < 0.05$). The V% of TB-positive goats were
156 significantly greater when stored as a solution than when lyophilized P22 was stored in both
157 temperatures. ELISAs made with serum samples from TB-positive sheep gave more
158 homogeneous results: V% did not differ significantly between assays made with the antigen in
159 solution or lyophilized and stored at 40 °C, or between assays with the antigenic solution stored
160 at 4 °C or with lyophilized antigen stored at 40 °C ($p < 0.05$). ELISAs of TB-positive alpacas
161 showed similar V% across all antigen storage conditions, although E% increased over time
162 (**Table 3**).

163 It is important to notice that no changes in the TB positive or negative status of each
164 serum sample from the production animals were found when the antigen was kept in different
165 temperatures or presented in an antigenic solution or lyophilized formulation.

166

167 **3.1.2 Wildlife species**

168 E% was more stable during the 60-day period for TB-negative wild boars (**Fig. 2**) than
169 for TB-negative badgers. **Table 2** shows that in ELISAs made with serum samples from TB-

170 negative badgers, V% was marginally smaller with antigen stored at 40 °C; however, V% did not
171 differ significantly between antigen stored as solution or lyophilized at the same temperature. In
172 ELISAs made with serum samples from TB-negative wild boars, V% was smaller when stored at
173 40 °C but not significantly different when comparing the different conditions. Furthermore,
174 **Table 3** shows that in ELISAs made with serum samples from TB-positive badgers, the V% was
175 marginally smaller when P22 was lyophilized and stored at 40 °C. TB-positive wild boars had a
176 significantly smaller V% when P22 was stored as solution at 40 °C. Generally, V% was greater
177 but not significantly different with antigen stored as solution than with the antigen stored
178 lyophilized.

179 In a same way that was observed for the production animal serum samples, the samples
180 taken from the wild animals did not have their TB positive or negative status altered when
181 antigen in different formulations or storage conditions were used at the indirect ELISA.

182

183 **3.2 Cross-reactivity with *Corynebacterium pseudotuberculosis* infection**

184 Sera from CLA-positive goats and sheep were evaluated using P22 ELISA, along with
185 control samples taken from TB culture-positive and negative animals (**Fig. 3**). None of the goats
186 with CLA were positive in the P22 ELISA. Regarding sheep, two animals (5,71%) with CLA
187 had positive results in the P22 ELISA (above the cut-off) (**Fig. 3**).

188 In order to analyze whether these two sheep were infected by the both pathogens or if the
189 P22 ELISA was giving false-positive results, a Western blot technique was performed. The two
190 animals positive for CLA recognized the *C. pseudotuberculosis* specific rPLD (lines 9 and 10,
191 **Fig. 4**), while no recognition was found when the same serum samples were incubated with the
192 P22 protein complex (**Fig. 4**, lines 4 and 5).

193

194 ## 4. Discussion

195 Diagnostic tests, particularly those for use in animal production units, should present a
196 robust performance, even when test components may not have been properly stored [10]. Our
197 study showed that an ELISA based on the P22 protein complex presented results (E%) consistent
198 with TB culture-based diagnosis in various animal species, independently of P22 storage
199 conditions. These results suggest that the P22-based ELISA has a significant stability and present
200 accurate results when used in an immunoenzymatic assay, even when the antigen is subjected to
201 different temperatures.

202 The P22 antigen is a complex containing MPB70, MPB83, ESAT-6, and CFP-10
203 glycoproteins [13], which present a variety of epitopes that may be differently recognized by
204 antibodies from different animal species [4-7]. The E% results were slightly lower for the
205 different species when antigen was stored at 40 °C than when it had been stored at 4 °C. This
206 probably reflects that a higher temperature leads to partial denaturation or degradation of the P22
207 complex. Nevertheless, enough epitopes (mainly linear epitopes) remained recognizable to
208 animal antibodies.

209 Lyophilization, when not properly conducted or in the absence of lyophilization
210 protective compounds, can permanently denature proteins, and they fail to recover their native
211 conformations upon rehydration [15]. Lyophilization can also lead to protein oxidation and
212 hydrolysis [15]. In these ways, lyophilization may reduce the number of epitopes that serum
213 antibodies can recognize. Nevertheless, we found that the P22-based ELISA provided an
214 adequate diagnostic performance even when the antigen was lyophilized. This situation can

215 reflect the number of proteins in the P22 complex, which helps to ensure that a minimum of
216 epitopes is accessible to serum antibodies, and without completely affecting ELISA results.

217 A significantly higher variance was found when serum samples from cows, goats and
218 alpacas were tested in the P22 ELISA, but this variance did not reach the validation criteria
219 (below 20%), and the results remained consistent with culture-based diagnosis. This
220 aforementioned fact could be related to the higher differences between positive and negative
221 results stablished at the ELISA's standardization [4-7], where negative results presented, in
222 average, a low E% (< 50), while positive results presented significantly higher E% values (>
223 400), and even large variances were not sufficient to change the final results.

224 Cross-reactivity can be defined as the presence of similar peptide epitopes within two or
225 more distinct proteins belonging to a phylogenetically related group of pathogens [16].
226 *Mycobacterium* and *Corynebacterium* genera are part of the CMNR (*Corynebacterium*,
227 *Mycobacterium*, *Nocardia* and *Rhodococcus*) group of pathogens, which is composed by bacteria
228 that are phylogenetically related and presents similar antigenic composition, mainly at the cell
229 wall and membrane [11]; therefore, they may share common antigenic factors (proteins and other
230 molecules) and cross-reactivity may occur in serological tests [11,17]. The cross-reactivity
231 among species belonging to the MTC is well established, and comparative tests were carried out
232 mostly in the specific cases of TB and paratuberculosis [4-7]. In particular, the P22 ELISA was
233 already submitted to cross-reactivity analysis with paratuberculosis and presented great
234 specificity results even when the flock was infected by this bacterium [6,9].

235 No cross-reaction was observed in this study when serum samples taken from 35 CLA-
236 positive goats, from TB non endemic areas, were tested in the P22 ELISA. However, when
237 considering the 35 sheep serum samples studied herein, a small percentage of cross-reactivity

238 (5.7%) was observed between the P22 ELISA and the diagnosis of CLA made through bacterial
239 isolation and by an indirect ELISA made with rPLD antigen [14], since serum samples taken
240 from 02 CLA-positive animals from an area where TB is not endemic presented positive results
241 at the P22 ELISA. This small level of cross-reactivity can be related to a heterogenous humoral
242 response that is elicited by the *C. pseudotuberculosis* infection in small ruminants, where a set of
243 bacterial antigens is differentially recognized by these animals, even when the animals present a
244 more homogenous genetic background [18]. The same situation was already seen for the cellular
245 immune response of goats and sheep elicited by the bacterium [19], where the specific IFN-
246 production induced by bacterial antigens was highly heterogenous, especially for sheep. In this
247 way, a small percentage of *C. pseudotuberculosis*-infected animals can develop a repertoire of
248 antibodies that can recognize the P22 antigen, even when not infected by *Mycobacterium* spp.

249 Most human autoantibodies recognize conformational epitopes rather than linear ones,
250 and immunodiagnostic methods such as ELISA exhibit low specificity for these auto-antigens
251 [20]. Regarding bacterial antigens, it was already described that the use of a heated *Brucella*
252 *canis* antigenic extract was able to confer a 100% specificity to an indirect ELISA, while the not-
253 heated antigenic extract showed an 84.31% specificity; these results were also associated with a
254 less intense cross-reactivity with leptospirosis, ehrlichiosis, babesiosis and distemper [21]. The
255 heating process can induce the loss of conformational epitopes, and in this way, it can reduce the
256 cross-reactions with antigens derived from other pathogenic agents. This situation was also
257 observed in the present study, since the Western Blot assay, that is characterized for using heated
258 denaturated antigens, did not present the cross-reactions that were seen for two CLA-positive
259 sheep in the P22 ELISA.

260 Cross-reactivities can be mitigated by heating the antigenic solution, but it was also seen
261 by de Oliveira and collaborators [21] that, besides an improvement in the specificity of the assay,
262 the use of the heated antigen caused a significant drop in its sensitivity. Another option that can
263 be taken in consideration is a pre-adsorption step, as previously described by a study that was
264 able to reduce the cross-reactivity with *C. pseudotuberculosis* in an assay designed to detect *M.*
265 *pseudotuberculosis* specific antibodies [17]. Consistent studies mainly focused on the cross-
266 reactivity between *M. tuberculosis* and *C. pseudotuberculosis* are still lacking; nonetheless,
267 Bezos and collaborators [12] conducted an analysis on a CLA-positive goat herd to assess cross-
268 reactions using single and comparative intradermal tuberculin (SIT and SCIT, respectively) tests
269 and specific IFN-γ release assay for TB, and found no cross-reaction related to the CLA-positive
270 status of the animals. Likewise, our study showed that when sera from CLA-positive goats are
271 tested on P22 ELISA, no cross-reactions were observed.
272
273 **5. Conclusions**
274 The results obtained herein suggest that P22 is a very stable protein complex, and
275 differences on its presentation and storage does not alter the serodiagnosis of TB in a variety of
276 domestic and wild species. Although a 5.7% (2/35) cross-reactivity was observed when sheep
277 sera was assessed, no cross-reaction was observed for goats, and therefore the P22 ELISA is a
278 reliable tool to correctly serodiagnose TB in small ruminants, even in CLA endemic areas.
279
280 **Author contributions**

281 TDB, JAI-L, IM, RM, RDP, LD, MD, and AB conceived and designed the study. All authors
282 performed experiments and analyzed the data. TDB drafted the manuscript, which all authors revised
283 and approved for submission.

284

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297

298 **Conflicts of interest**

299 The authors declare no conflicts of interest.

300

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384

385 **Tables**

386 **Table 1**. Serum samples used in the present study. These samples (stored at -20 ºC) were
387 available from the animal serum collections at the Universidad de León (León, Spain), the
388 Instituto de Salud Carlos III (Madrid, Spain) and the Federal University of Bahia (Salvador,
389 Brazil).

Species	Positive at the *M. tuberculosis* isolation (n)	Positive at the *C. pseudotuberculosis* isolation (n)	Negative at bacterial isolation (n)
Cow	1	-	5
Goat	26	35	35
Sheep	6	35	35
Alpaca	3	-	3
Badger	1	-	5
Wild boar	6	-	6

390 n: number of sera.

391

392 **Table 2**. E% variance during the 60-day evaluation period, as presented by the P22-based
393 ELISAs using serum samples from TB-negative animals. The results are stratified by
394 presentation (solution or lyophilized) and storage conditions (4 °C or 40 °C) of the P22 protein
395 complex antigen.

Species	Variance of percent value (%)			
	Antigen solution		Lyophilized antigen	
	4 °C	40 °C	4 °C	40 °C
Cow	4.6	3.8	7.6	8.9
Goat	14.7	19	3.1	12.2
Sheep	3.5	3.5	5.2	10.5
Alpaca	19.8	16.9	27.7*	24.5*
Badger	6.9	12.8	11.6	7.7
Wild boar	5.6	4.1	6.9	4.6

396

397 **Table 3**. E% variance during the 60-day evaluation period, as presented by the P22-based
398 ELISAs using serum samples from TB-positive animals. The results are stratified by presentation
399 (solution or lyophilized) and storage conditions (4 °C or 40 °C) of the P22 protein complex
400 antigen.

Species	**Variance of E% (%)**			
	Antigen solution		**Lyophilized antigen**	
	4 °C	**40 °C**	**4 °C**	**40 °C**
Cow	14.9	23.4*	9	7.8
Goat	24.7*	39.8*	10.5	9.2
Sheep	3.7	13.6	8.2	7.6
Alpaca	16.1	22*	19	27.1*
Badger	6.2	13.9	7.6	7.1
Wild boar	10.7	19.1	5.6	2.7

401

402 **Figure legends**

403 **Figure 1.** P22 ELISAs made with serum samples from TB-positive and -negative production
404 animals (cow, sheep, goats and alpacas). The P22 protein was used as a solution or lyophilized
405 and maintained at 4 °C or 40 °C, and then applied at the indirect ELISA. Results are expressed in
406 ELISA percentage (E%), calculated as described in the Material and methods section.

407

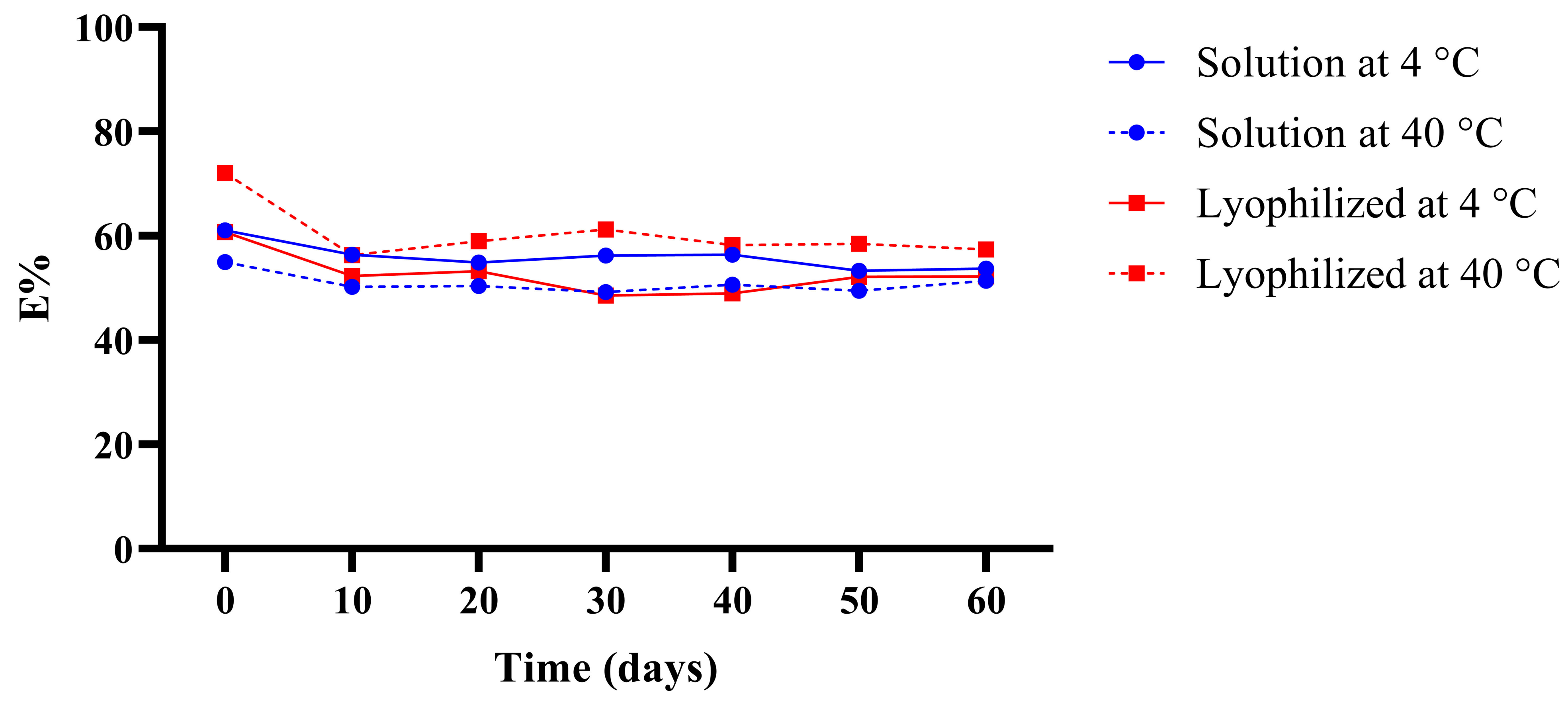
408 **Figure 2.** P22 ELISAs made with serum samples from TB -positive and -negative wild animals
409 (badgers and wild boars). The P22 protein was used as a solution or lyophilized and maintained
410 at 4 °C or 40 °C, and then applied at the indirect ELISA. Results are expressed in ELISA
411 percentage (E%), calculated as described in the Material and methods section.

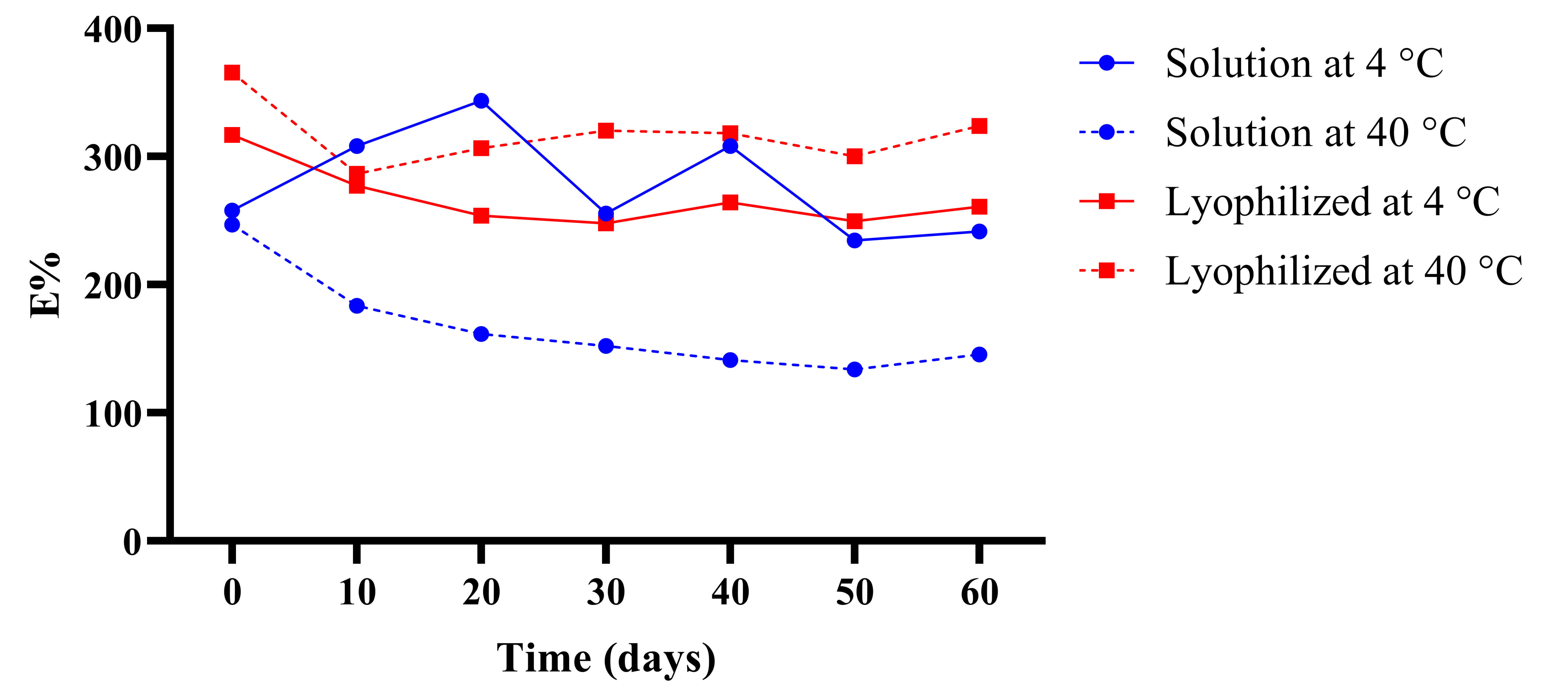
412

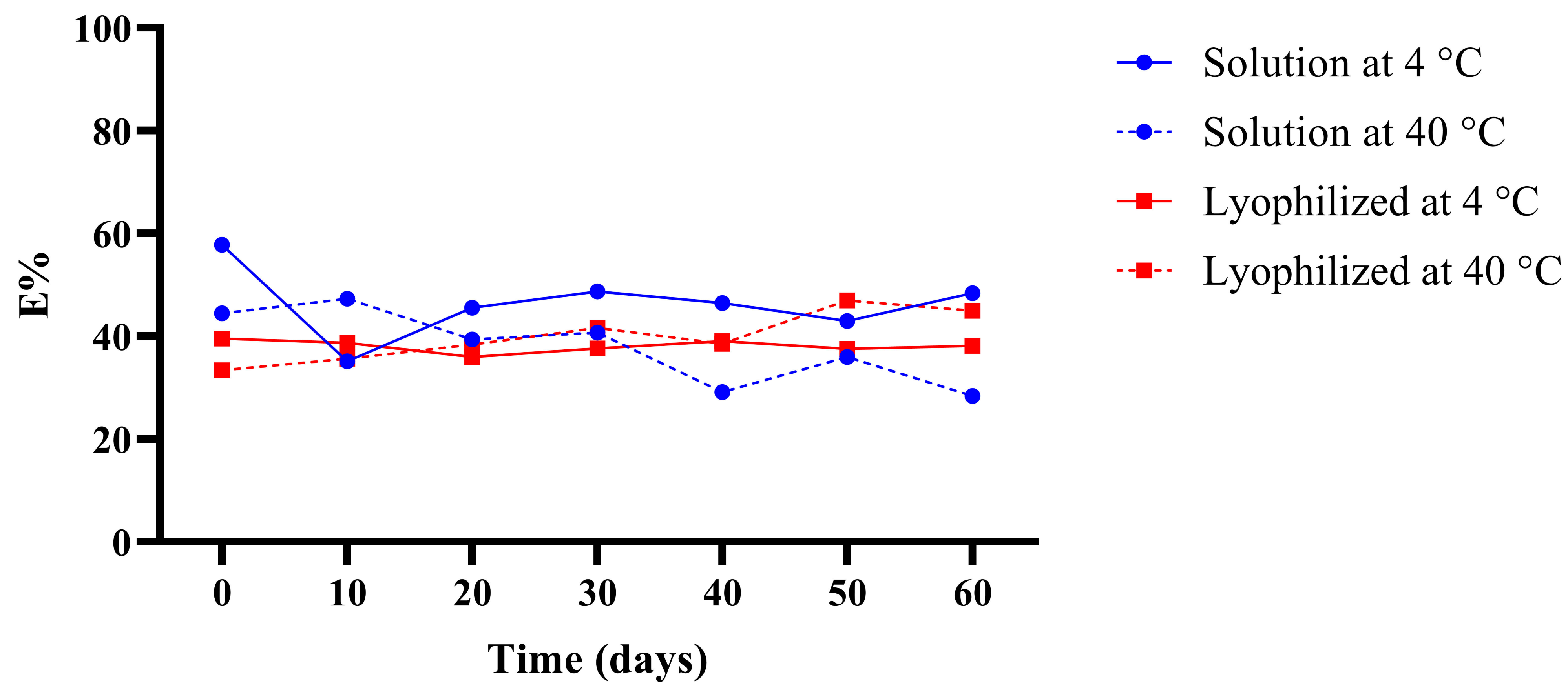
413 **Figure 3.** Individual results obtained using P22 ELISA and serum samples from TB positive, and
414 CLA positive and negative sheep and goats. The line within the graphs represents the cut-off
415 point. The red circle emphasizes the two CLA-positive samples that presented positive results at
416 the P22 ELISA. Results are expressed in ELISA percentage (E%), as described in the Materials
417 and methods Section.

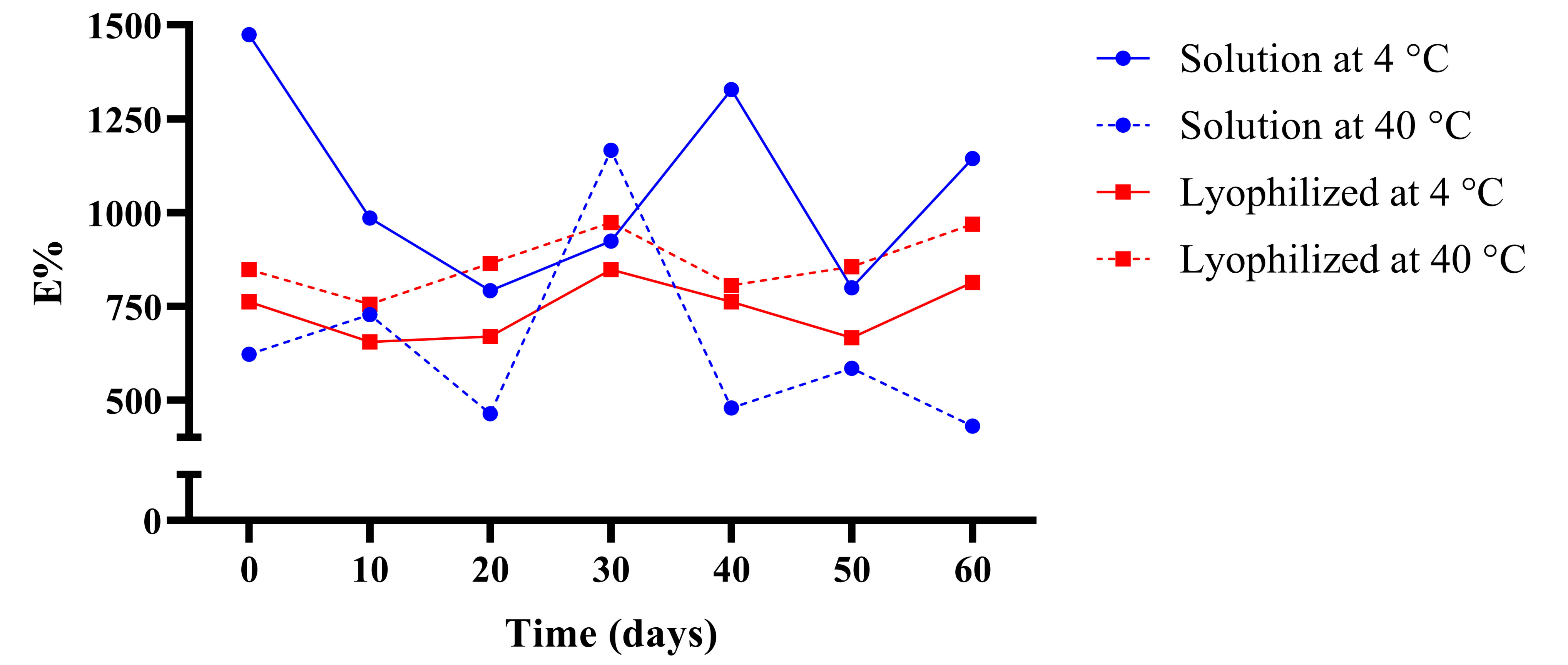
418

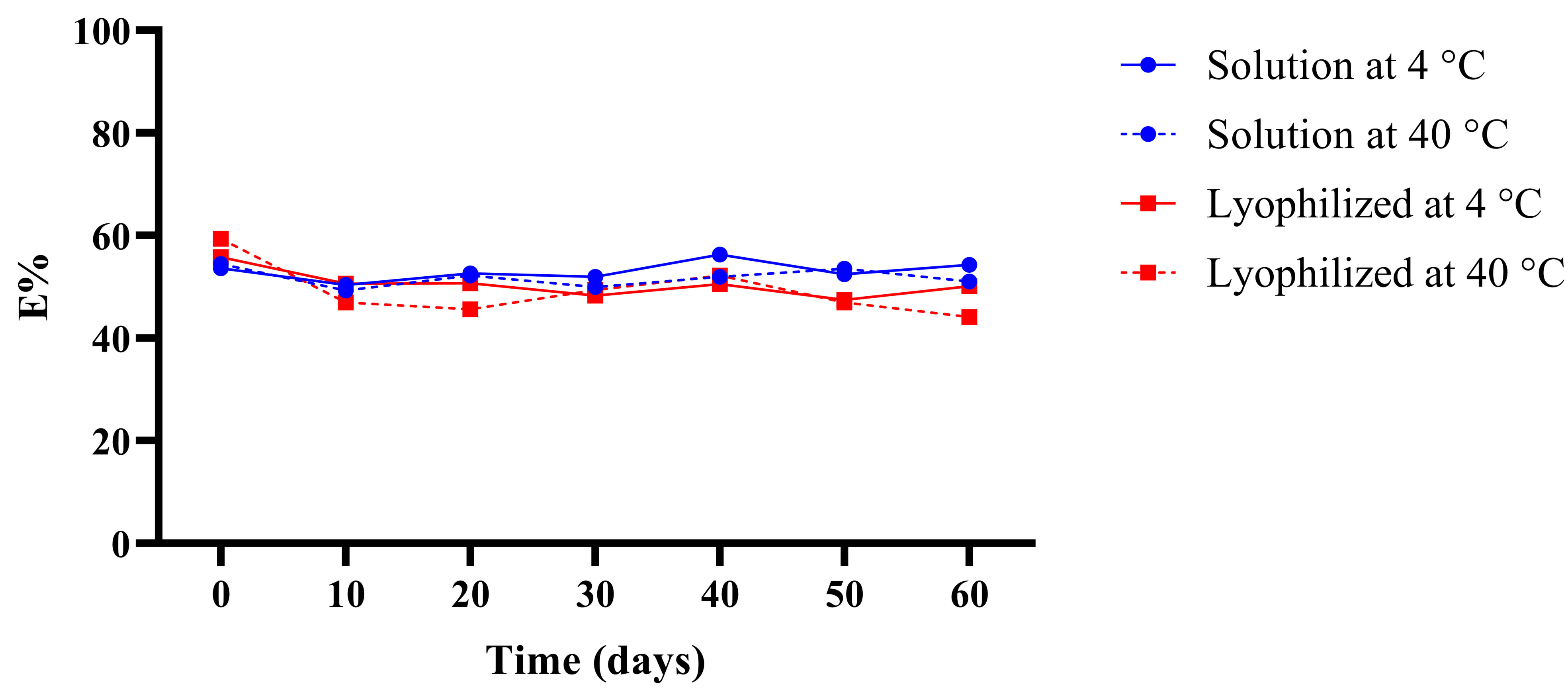
419 **Figure 4.** Western blot assays using the *Mycobacterium* sp. P22 protein complex (lines 1-5) or
420 the *C. pseudotuberculosis* rPLD (lines 6-10) as antigens. Different sheep serum samples were
421 incubated with the antigens: 1 and 6 – TB positive control; 2 and 7 – CLA positive control; 3 and
422 8 – negative control; 4 and 9, 5 and 10 – two CLA positive sheep presenting cross-reactivity on
423 P22 ELISA. S – molecular weight standard (Thermo Scientific, ref #26619). The numbers at the
424 side represent the molecular weight, in kDa.

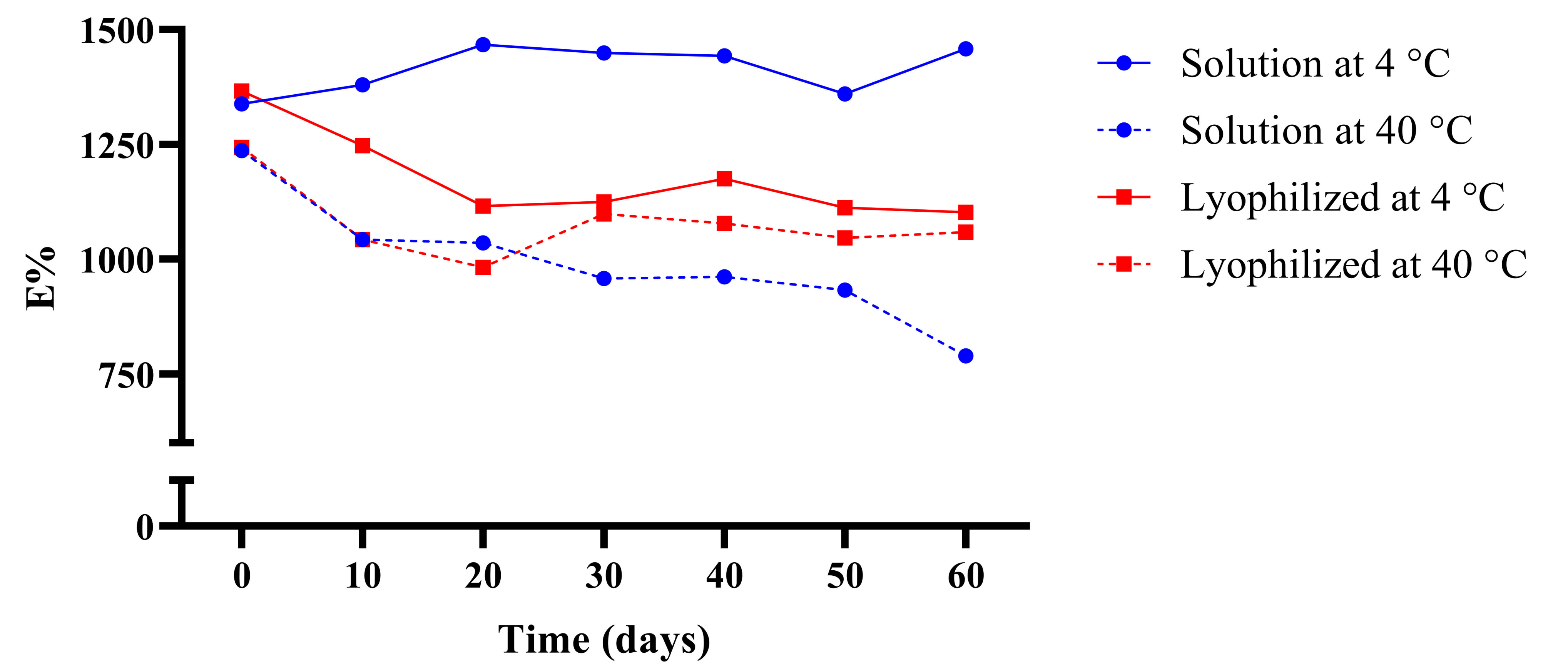

TB-negative Cow
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

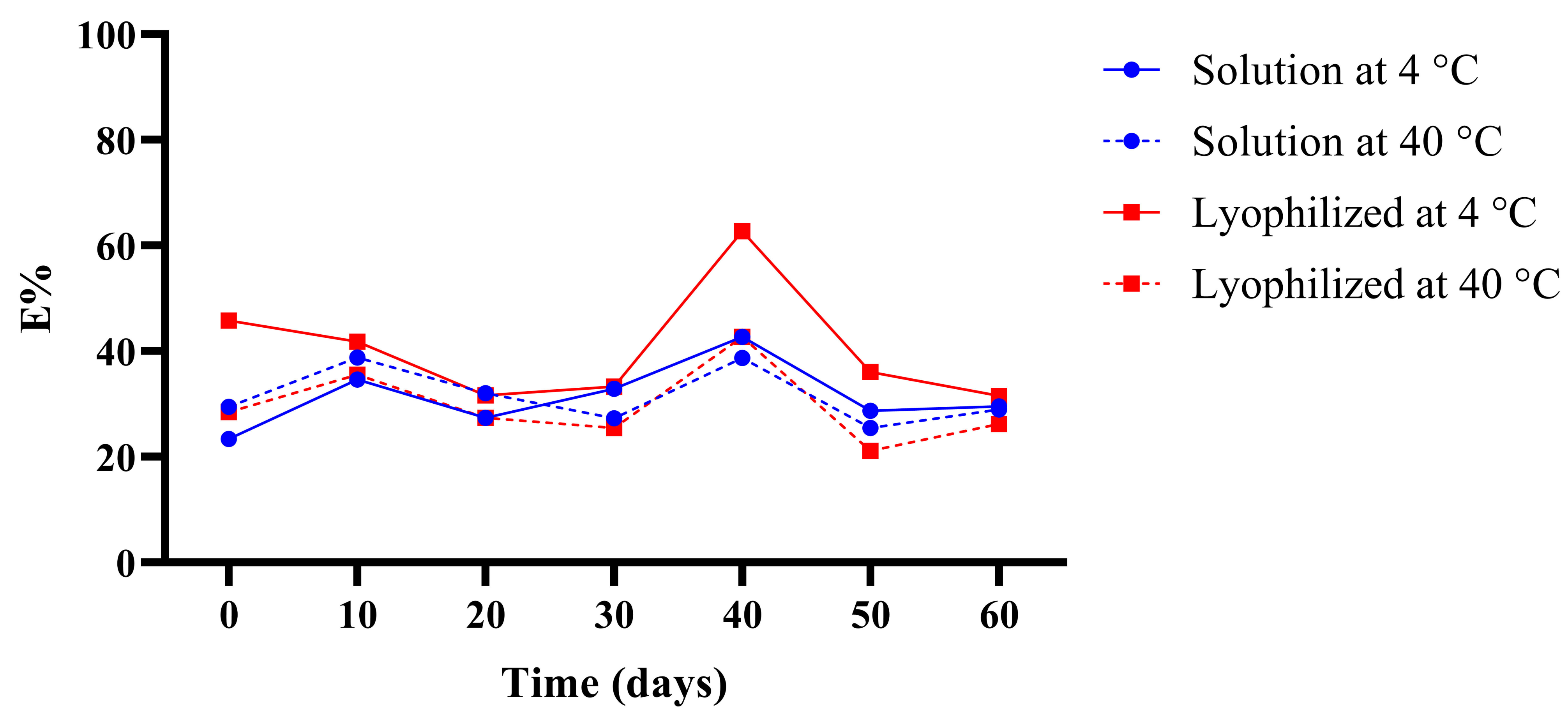

TB-positive Cow
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

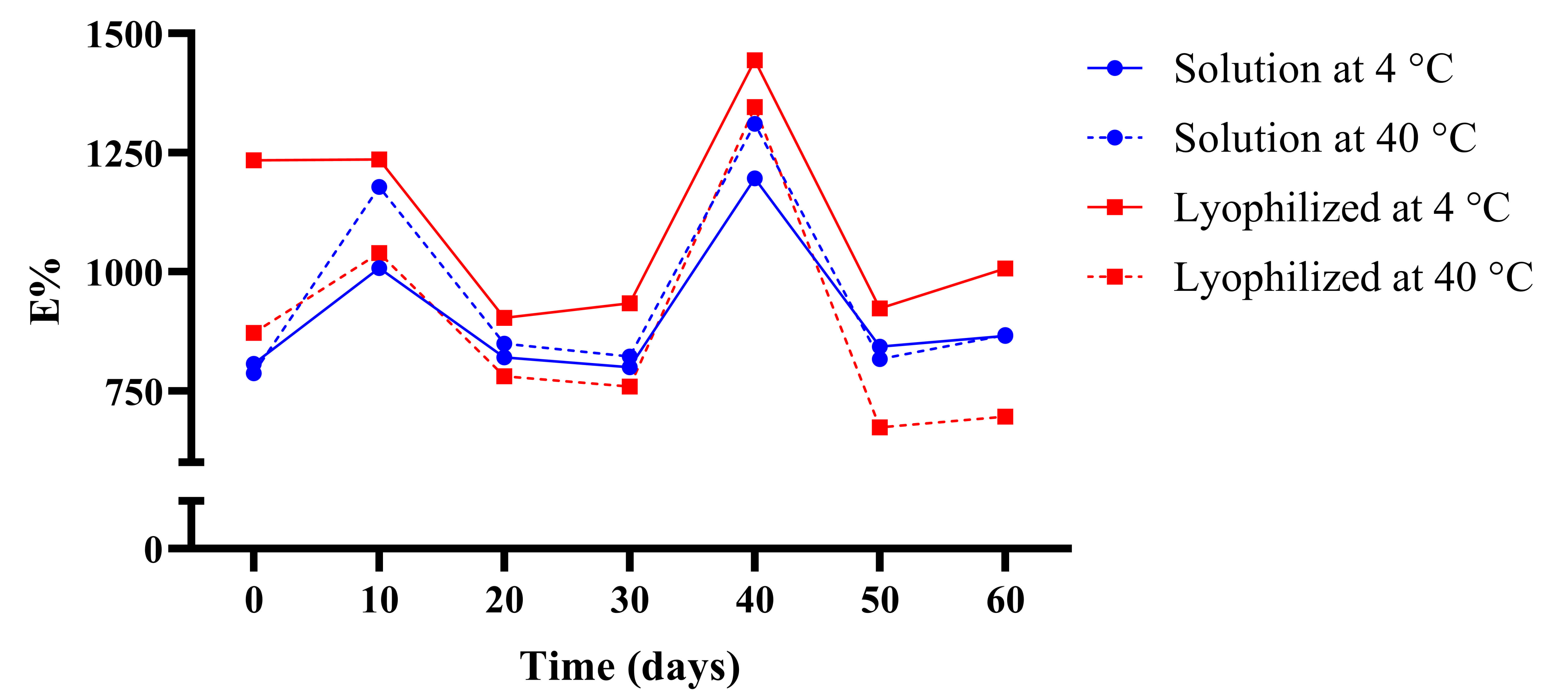

TB-negative Goat
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

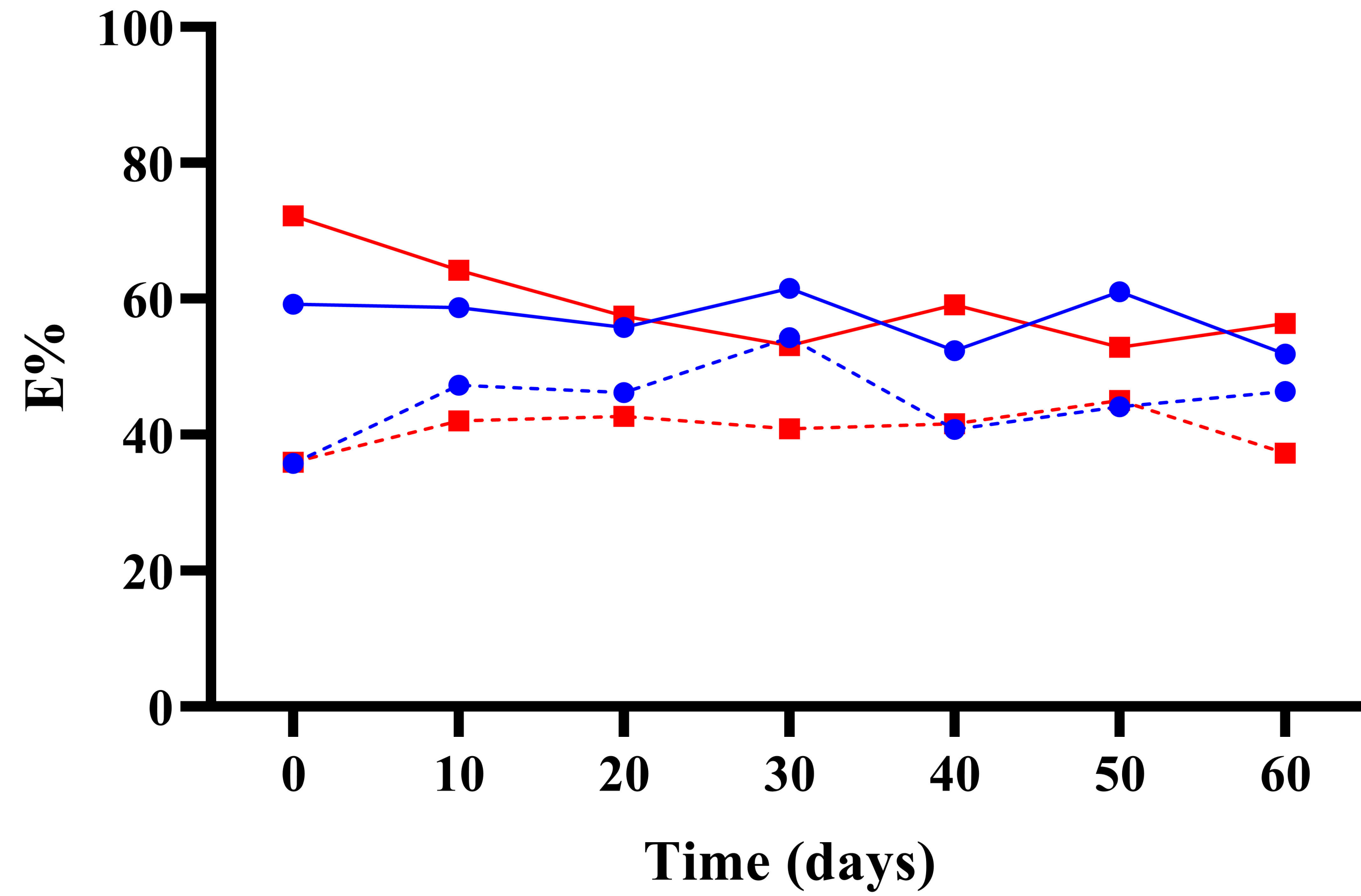

TB-positive Goat
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

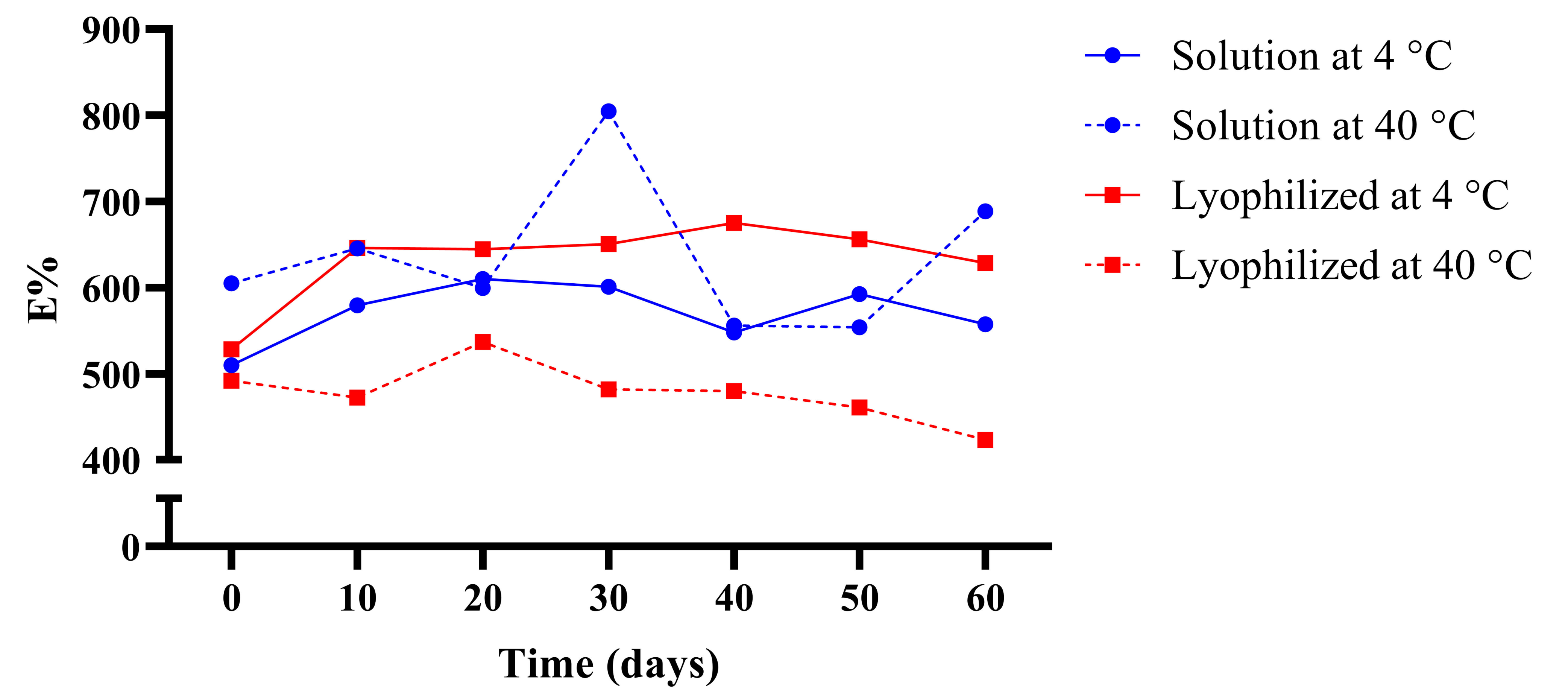

TB-negative Sheep
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

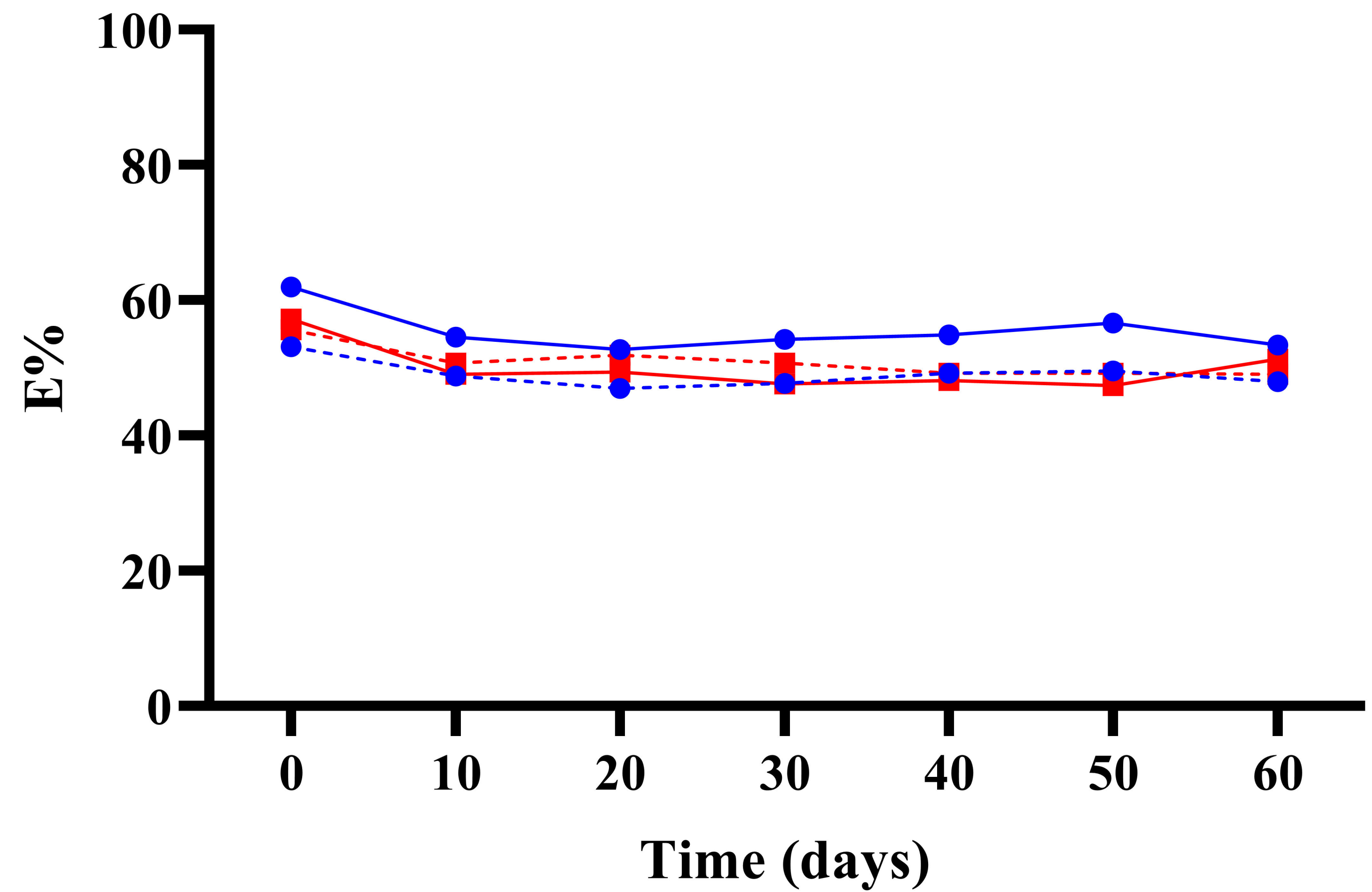

TB-positive Sheep
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

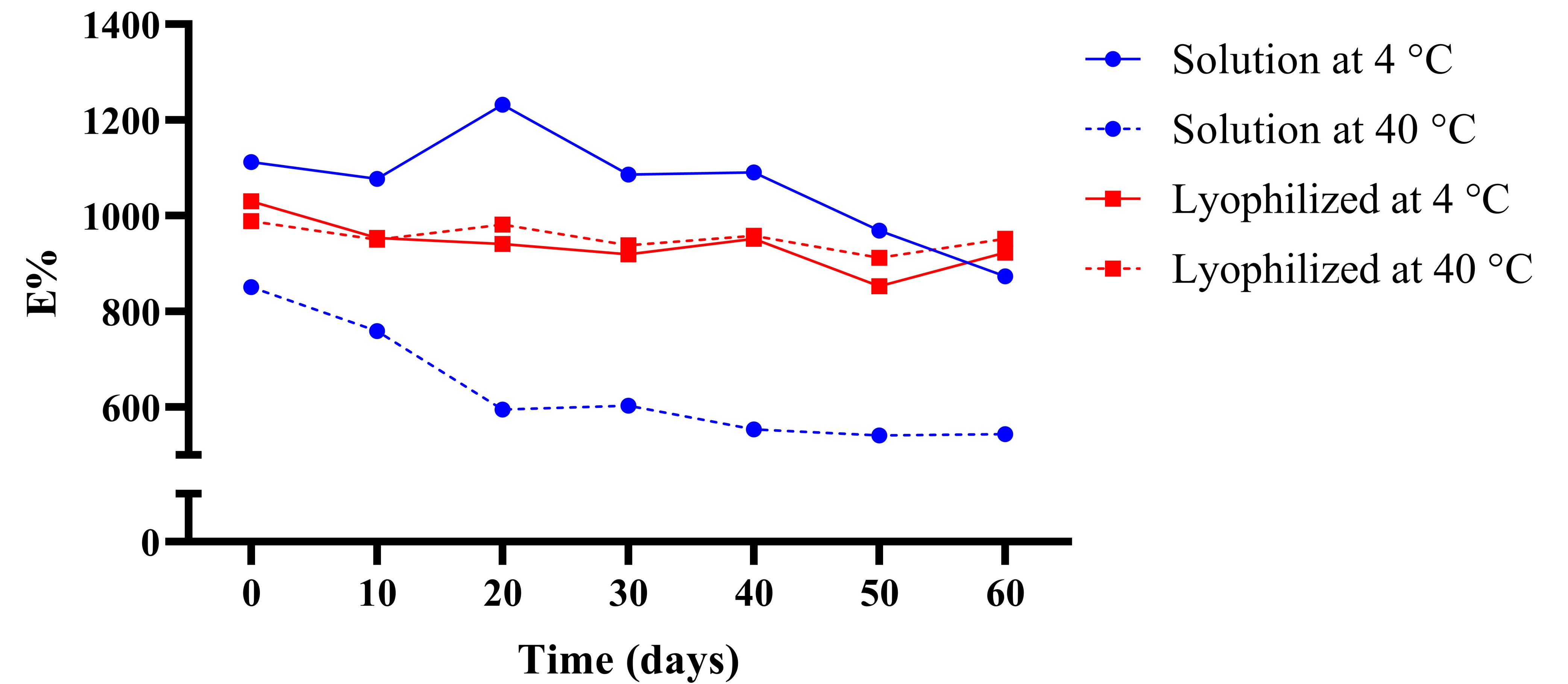

TB-negative Alpaca
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C


TB-positive Alpaca
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C


TB-negative Badger
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C


TB-positive Badger
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C


TB-negative Wild boar
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C


TB-positive Wild boar
E%
Time (days)
Solution at 4 °C
Solution at 40 °C
Lyophilized at 4 °C
Lyophilized at 40 °C

P22 ELISA for Goat

3000
2500
2000
1500
1000
500
0

E%

TB Positive
n = 26

CLA Posistive
n = 35

Negative
n = 35

P22 ELISA for Sheep

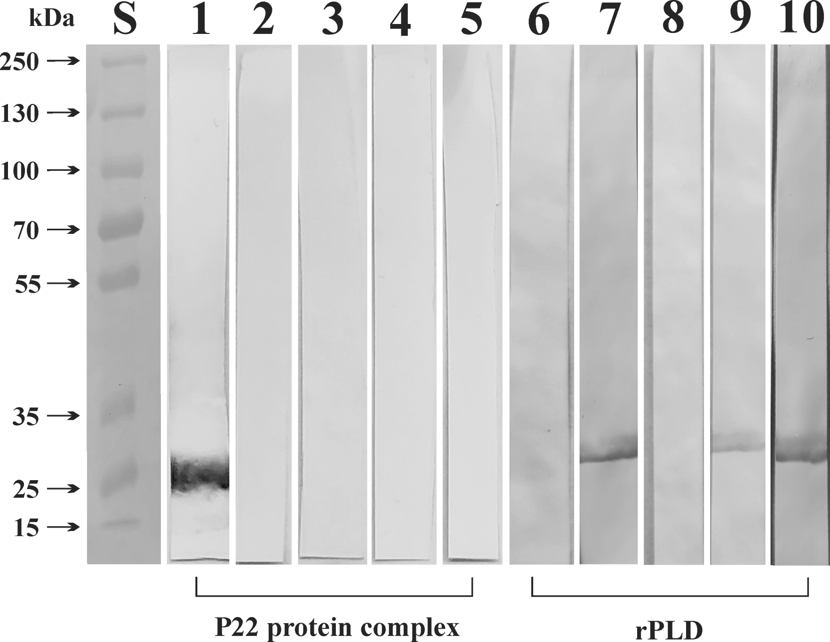
3000
2500
2000
1500
1000
500
0

E%

TB Positive
n = 6

CLA Posistive
n = 35

Negative
n = 35


kDa
S
1
2
3
4
5
6
7
8
9
10
250
130
100
70
55
35
25
15
P22 protein complex
rPLD