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► **To cite this version:**

Marine Leroux, Uruguaysito Benavides, Imène Hellel-Bourtal, Cecilia Silvarrey, Nadège Milhau, et al.. Development of an oral nanovaccine for dogs against *Echinococcus granulosus*. *European Journal of Pharmaceutics and Biopharmaceutics*, 2023, 192, pp.185-195. 10.1016/j.ejpb.2023.09.012 . hal-04504162

HAL Id: hal-04504162

<https://univ-lyon1.hal.science/hal-04504162>

Submitted on 18 Mar 2024

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1 Running header: *Echinococcus* nanovaccine development

2 **Development of an oral nanovaccine for dogs against *Echinococcus granulosus***

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17

18 **Key words**

19 Polymeric nanoparticles; Chitosan, *Echinococcus granulosus*; Oral vaccine, Dog

20

21 **Abstract**

22 Dogs are the main source of animal and human cystic echinococcosis caused by the Cestode
23 parasite *Echinococcus granulosus*. Dog vaccination seems to be a good strategy to control this
24 parasitic disease. Here we present the development of a polymeric nanoparticle-based oral
25 vaccine for dogs against *Echinococcus granulosus* delivered in enteric-coated capsules. To
26 achieve our target, we encapsulated two recombinant antigens into biodegradable polymeric
27 nanoparticles in the presence of Monophosphoryl lipid A as an adjuvant to ensure efficient delivery
28 and activation of a protective mucosal immune response. The formulated delivery system showed
29 a nanoparticle size less than 200 nm with more than 80% antigen encapsulation efficiency and

30 conserved integrity and immunogenicity. The nanoparticle surface was coated with chitosan to
31 enhance adhesion to the gut mucosa and a subsequent antigen delivery. Chitosan-coated
32 nanoparticles showed a higher cell internalization in murine macrophages and dendritic cells as
33 well as a higher penetration into Caco-2 cells in vitro. Antigen-loaded nanoparticles were freeze-
34 dried and enteric-coated capsules were filled with the obtained powder. The obtained results show
35 a promising nanoparticles delivery system for oral vaccination.

36

37 **1. Introduction**

38 Hydatid infection caused by the cestode *Echinococcus granulosus* (*E. granulosus*) is still one of the
39 major zoonoses, affecting humans as well as domestic and wild animals worldwide (Thompson &
40 Jenkins, 2014). The parasite requires two mammalian hosts to complete its life cycle. The adult
41 worm develops in the intestine of the definitive host (dogs), and the eggs are shed with the host's
42 feces. They are able to infect a wide variety of intermediate hosts (herbivorous and omnivorous
43 animals) including humans that are accidentally infected. The development of the eggs into larval
44 stages throughout the internal organs, especially the liver and lungs, causes Hydatid Disease. *E.*
45 *granulosus* is distributed worldwide and the WHO has estimated that more than 1 million people
46 are affected by echinococcosis at any one time for around US\$ 3 billion of annual costs for treating
47 cases and losses to the livestock industry. Control programs developed in several countries using
48 antiparasitic drugs in sheep or dogs gave limited results in stopping the infection and the disease's
49 frequency (Torgerson & Heath, 2003).

50 Vaccination could be the most effective strategy to control this infection. Vaccination of dogs, the
51 main definitive hosts, may provide significant clinical and epidemical outcomes by arresting the
52 development of adult tapeworms (Pourceif et al., 2018). Several authors have demonstrated that
53 dogs can develop a protective immunity against *E. granulosus* through repetitive natural infections
54 (Gemell, 1962; Movsesijan et al., 1968) or by immunization with native proteins (Zhang et al.,
55 2006) suggesting that it is possible to develop a vaccine using parasite recombinant antigens to
56 develop protective immunity.

57 Vaccination at the site of infection is highly desirable to obtain a local mucosal defense at the entry
58 pathways of pathogens. Indeed, mucosal delivery is the only vaccination route that induces both
59 mucosal and systemic immune responses, especially if the vaccine contains adjuvants,
60 immunostimulators or carriers. Locally produced secretory IgA constitute over 80% of all antibodies
61 produced in mucosa-associated tissues and are considered to be among the most important
62 protective humoral immune factors. However, induction of a robust protective immune response by
63 oral immunization requires successful delivery of the intact and active antigen to Antigen
64 Presenting Cells (APCs) of the intestine mucosa (Vela Ramirez et al., 2017). To be effective, the
65 antigen delivery system must: (i) protect the antigen from the highly acidic environment of the
66 stomach (pH 1-3) and from the presence in the duodenum and jejunum of proteolytic enzymes
67 responsible for protein degradation, (ii) ensure transport of the antigen across the mucosal barrier
68 to APCs, and (iii), enhance via adjuvants the elicited immune responses.

69 In previous works, our team identified two antigens *EgTrp* (*E. granulosus* tropomyosin) and *EgA31*
70 (*E. granulosus* paramyosin) as potential vaccine candidates (Esteves et al., 2003; Fu et al., 1999).
71 These recombinant antigens were tested for their immunogenic properties confirming the interest
72 to use them for future vaccine development (Saboulard et al., 2003; Pétavy et al., 2008).
73 Furthermore, our first vaccination trials in dogs using these recombinant proteins administered
74 orally induced a high level of protection with high immunogenicity and decreased the parasite
75 burden by more than 70% (Pétavy et al., 2008). This vaccine system using an attenuated
76 *Salmonella* delivery system expressing both recombinant antigens could be considered as an
77 environmental problem (Karch & Burkhard, 2016). For these reasons, developing a safer
78 alternative vaccine delivery system for these recombinant antigens, suitable for dog oral
79 administration could be an interesting strategy in the control plans against *E. granulosus*.

80 Biodegradable polymeric nanoparticles (NPs) have been described as a potential antigen delivery
81 system for systemic and mucosal vaccine development (Zhao et al., 2014) and hence, we decided
82 to develop a robust antigen delivery system based on NPs. **As a matter of fact, polymeric particles**
83 **are promising vehicles for orally antigen delivery because their physicochemical properties can be**
84 **adapted to the particular environment of the gastro-intestinal tract.** We previously reported the

85 feasibility to encapsulate *EgTrp* as antigen and the monophosphoryl lipid A (MPLA) as adjuvant in
86 polymeric NPs. The developed system was evaluated *in vitro* on canine monocyte-derived
87 dendritic cells (cMoDC) confirming the ability of such a system to deliver the antigen and induce
88 cMoDC maturation and autologous T CD4+ proliferation more efficiently than the antigen alone
89 (Milhau et al., 2020).

90 In the present work, we report the design of an oral vaccine delivery system using MPLA-NPs
91 coated with chitosan (CS-MPLA-NPs) loaded with the two recombinant antigens (*EgTrp* and
92 *EgA31*) included in enteric-coated capsules to protect the freeze-dried antigen-loaded CS-MPLA-
93 NPs. The interactions of this nanoparticle delivery system were evaluated on Caco-2 cells (a
94 human intestinal cell line), mouse macrophages (J774A cell line) and mouse dendritic cells to
95 observe the effect of chitosan.

96 Chitosan is a cationic polysaccharide with advantageous properties for oral delivery, including
97 enhanced mucoadhesion through interactions between the positive charges of the polymer and the
98 negatively charged intestinal cells membrane, and an ability to facilitate antigen transport across
99 the mucosal barrier either through a paracellular route by a reversible disruption of epithelial tight
100 junctions or via transcytosis through enterocytes (Xia et al., 2015). However, chitosan coating is
101 limited by its high solubility in acidic conditions and must be protected from gastric hyper-acidity.
102 Consequently, we tested different enteric-coated capsules to complete this formulation. Enteric-
103 coated capsules must protect NPs from the highly acidic conditions of the stomach but must be
104 rapidly dissolved in the less acidic condition of the duodenum to release NPs and allow chitosan to
105 exert its effects.

106

107 **2. Materials and methods**

108 **2.1. Vaccine formulation**

109 **2.1.1. Antigen production and purification**

110 The recombinant *EgTrp* antigen was produced as previously described (Milhau et al., 2020).
111 *pQIA-EgTrp* was expressed in M15 (pREP4) (Qiagen) *Escherichia coli* strain. Bacterial cells
112 containing the relevant expression plasmid were cultured at 37°C in yeast extract and tryptone
113 media supplemented with ampicillin/kanamycin, up to 600 nm. *EgTrp* expression was induced by

114 the addition of 1.5 mM isopropyl β -D-1-thiogalactopyranoside and incubated for further 3 h. After
115 centrifugation at 5,000 rpm for 25 min, the cells were collected and re-suspended in cell lysis
116 buffer (50 mM phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole) and ruptured by sonication.
117 Cellular debris were removed by centrifugation at 15,000 x g for 30 min at 4°C, and the
118 supernatant was loaded onto His-Trap column (GE Healthcare, Pasching, Austria). Elution was
119 performed in the same lysis buffer with increasing concentrations of imidazole (100 mM, 200 mM,
120 300 mM, 500mM).

121 The *EgA31* coding region cloned into the vector pET22b (NEB) was expressed in the
122 *Escherichia coli* BL21 strain and the antigen was subsequently purified according to the same
123 protocol as the one previously described for *EgTrp*, except that 8 M urea was used for the lysis and
124 elution buffers. The eluted fractions were analyzed by SDS-PAGE using the PageRuler Prestained
125 Protein Ladder (0 to 170 kDa - Thermo Scientific) as molecular weight markers and analysed by
126 MALDI-TOF mass spectrometry.

127

128 **2.1.2. Preparation of recombinant antigen-loaded nanoparticles**

129 The two recombinant antigens were encapsulated separately into polymeric nanoparticles
130 using the emulsion-diffusion-solvent evaporation method as described in our previous article
131 (Milhau et al., 2020). First, poly (D,L) lactic acid (PLA) (Mw 20000, Evonik Biomaterials) was
132 dissolved in 20 mL ethyl acetate at a final concentration of 5 %. The adjuvant MPLA
133 (Monophosphoryl-lipid A from *Salmonella enterica* serotype Minnesota Re 595, Sigma-Aldrich, St-
134 Quentin Fallavier, France) previously solubilized in a methanol-chloroform mixture (1:4 v/v) was
135 then introduced into the organic phase with magnetic stirring. The first emulsion was made by
136 adding dropwise the internal aqueous phase (about 4 mg of *EgTrp* or *EgA31* in 2 mL of their
137 elution buffers) to the organic phase under magnetic stirring combined with sonication at a 25 %
138 amplitude (S-4000, Misonix) for 2 minutes. The second emulsion was made by dropping the first
139 emulsion in 40 ml of a polyvinyl alcohol (PVA, 31000 MW, Sigma-Aldrich, St-Quentin Fallavier,
140 France) aqueous solution (2%) in the same conditions for another 2 min on ice. The final
141 preparation was stirred for 20 minutes. Finally, the organic solvent was evaporated at 40°C and
142 200 mBar using a rotary evaporator (Rotavapor® RE-140, Büchi, Switzerland). The final

143 nanoparticle suspension was adjusted to 40 ml final volume with demineralized water and was
144 stored at 4°C for further use. Blank-MPLA-NPs were prepared in similar conditions by replacing the
145 antigen and the elution buffer by 2 mL of distilled water. For the evaluation of NPs internalization,
146 Nile red (Sigma-Aldrich, St-Quentin Fallavier, France), a fluorescent marker, was added (4 mg of
147 Nile red per gram of PLA) to the organic phase and these Nile red marked nanoparticles (NR-
148 MPLA-NPs) were prepared as described above.

149

150 **2.1.3. Preparation of Chitosan-coated nanoparticles**

151 In order to prepare Chitosan-coated nanoparticles (CS-MPLA-NPs), following organic
152 solvent evaporation, NPs were treated as described by Wang et al.,(2013) with a modification of
153 the operative conditions. The NP suspension was first centrifuged at 28000 g for 15 min and the
154 supernatant was decanted to eliminate the PVA. NPs were then suspended in a hydrochloride acid
155 aqueous solution of CS (deacetylation \geq 75%, Sigma-Aldrich) and kept under magnetic stirring for
156 different incubation periods. The suspension was then centrifuged at 28000 g for 15 min, the
157 supernatant was decanted to eliminate excess of CS and finally CS-MPLA-NPs were suspended in
158 deionized water. Different chitosan concentrations (0.025 – 0.1%) and incubation periods (1, 4 or
159 24 h) were tested in order to obtain the optimal positive charge of NPs. The NPs used for *in vitro*
160 and *in vivo* evaluations were prepared with the selected conditions using 0.1% of CS solution and
161 4 h of incubation.

162

163 **2.1.4. NP freeze-drying and capsule filling**

164 To ensure long term stability, CS-*EgTrp*-MPLA-NPs and CS-*EgA31*-MPLA-NPs were
165 lyophilized and capsule-filled separately in order to avoid stability problems. Mannitol was added to
166 the NP suspension at a final concentration of 2% as a lyoprotectant and a bulking agent. The NP
167 suspension was frozen for 3 hours at -80°C before the sublimation step. Lyophilization was
168 performed in laboratory-scale freeze-dryer (Alpha, Christ) at a shelf-temperature of -15 °C and a
169 pressure of 500 μ Bar for 20 h. Hard capsules were then filled with the lyophilized NP containing
170 250 μ g of antigen per capsule and kept at room temperature. For this step two types of hard
171 capsules were tested: hard gelatin capsules (Capsugel®) and hypromellose

172 (hydroxypropylmethylcellulose) capsules containing gellan gum (DRcaps, Capsugel®). Finally,
173 enteric-coating was performed by immersing the capsules in ethanol containing 6% of an anionic
174 copolymer based on methacrylic acid and ethyl acrylate (EUDRAGIT® L100-55). The disintegration
175 and dissolution assays were performed as described in the European Pharmacopeia at acidic (pH
176 1.2) and alkaline (pH 6.8) conditions.

177

178 **2.2. Nanoparticle characterization**

179 **2.2.1. Average size, zeta potential and morphology**

180 The NPs average size, size distribution and zeta potential in an aqueous environment were
181 investigated by dynamic light scattering and laser microelectrophoresis using the Zetasizer Nano
182 ZS90 (Malvern Instruments, UK) at 25°C. Nanoparticles' morphology was observed by
183 transmission electron microscopy (TEM) using a CM 120 microscope (Philips, Eindhoven,
184 Netherlands) operating at an accelerating voltage of 80 KV. A drop of NP suspension was placed
185 on a copper grid and stained by 1% of sodium silicotungstate in deionized water.

186

187 **2.2.2. Antigen encapsulation efficiency**

188 To evaluate the encapsulation efficiency, the NP suspension was centrifuged (28000 g for
189 30 min) in order to separate the free antigen from the encapsulated one. Afterwards, the total
190 amount of protein in the total formulation (T), the unencapsulated-protein in the supernatant (SN)
191 and the encapsulated-protein in the pellet (P) were compared. Acetonitrile was used to dissolve the
192 NP polymer and to extract the antigen. The protein obtained after this procedure was quantified
193 with the Bradford reagent (Sigma-Aldrich, St-Quentin Fallavier, France). The encapsulation
194 efficiency (EE) was then calculated as the percentage of the protein amount found in the pellet
195 compared to the protein amount added to the process.

196

197 **2.2.3 Antigen integrity**

198 The extracts from the NP suspension, the supernatant and the pellet were also analyzed by
199 12.5 % SDS-PAGE stained with Coomassie Blue to assess for their integrity. Recombinant *EgTrp*

200 and *EgA31* treated or not with acetonitrile were used as controls. For Western Blot analysis, an
201 anti-*EgTrp* rabbit immune serum was used.

202

203 **2.3. *In vitro* evaluation of interactions between nanoparticles and the gastro-intestinal** 204 **environment**

205 **2.3.1. *In vitro* tests in gastric and intestinal pH conditions**

206 The encapsulated-antigen integrity in gastrointestinal fluids, was evaluated on antigen-
207 loaded MPLA-NP suspensions. Nanoparticles were incubated in an aqueous solution at pH 1.6
208 and pH 6.8 simulating the gastric and intestinal conditions for 2 and 6 hours, respectively. MPLA-
209 NPs, were also incubated for 2 hours in an artificial gastric environment containing pepsin (SGF)
210 prepared according to the European Pharmacopeia recommendations. **As described in paragraph**
211 **2.2.2, the NPs were analyzed by 12.5 % SDS-PAGE stained with Coomassie Blue to assess for**
212 **their integrity.**

213

214 **2.3.2. Interactions with Caco-2 cells**

215 Caco-2 cells were seeded in a 6-well microplate at a density of 6.10^5 cells/well in
216 Dulbecco's Modified Eagle's Medium – high glucose containing 10% (v/v) fetal bovine serum
217 (Sigma, St-Quentin Fallavier, France), 1% MycoKill AB (GE healthcare, Pasching, Austria) and 2 %
218 Penicillin / Streptomycin (Dutcher, France) and incubated overnight at 37°C with 5% CO₂. After 24
219 hours, they were incubated with NR-MPLA-NPs and CS-NR-MPLA-NPs at a final 1:100 dilution
220 and observed by confocal laser scanning microscopy (Zeiss Axiovert 200M). For transmission
221 electron microscopy, the cells were fixed with 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4,
222 post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer,
223 dehydrated in a graded series of acetone and embedded in Epon resin. Ultrathin sections were
224 stained with uranyl acetate and lead citrate and observed under a Jeol 1400 JEM transmission
225 electron microscope (Tokyo, Japan) set at 120 kV.

226

227 **2.3.3. Interactions with mouse APC**

228 **2.3.3.1. Murine macrophages**

229 The cytotoxicity of NP suspensions was tested on mouse J774A macrophages using the
230 Cell-Titer 96 non-radioactive Cell Proliferation Assay (MTT) kit (Promega, Madison, WI, USA). The
231 BALB/c mice cell line J774A.1 (European Collection of Cell Culture) was maintained at 37°C with
232 5% CO₂ in RPMI-1640 (Dutscher, France) containing 10% (v/v) fetal bovine serum (Sigma, St-
233 Quentin Fallavier, France), 1% MycoKill AB (GE healthcare, Pasching, Austria) and 1.5% Penicillin
234 / Streptomycin (Dutscher, France). They were seeded in 96-well microplates in 50 µL of Minimal
235 Essential Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine
236 Serum (Life Technologies) and incubated overnight at 37°C with 5% CO₂ until the cells covered 80
237 to 90 % of the bottom of the well. After that, the macrophages were incubated for 24 hours with
238 several formulation dilutions ranging from 1:10 to 1:1000.

239 **For uptake studies**, NR-MPLA-NPs and CS-NR-MPLA-NPs were used to track their
240 internalization inside macrophages with a final dilution of 1:100. The macrophages were incubated
241 for 2h with NPs and the medium was replaced. After 24h, the samples were counterstained with
242 the nucleus-specific Hoechst 33342 fluorescent dye and observed by confocal laser scanning
243 microscopy (Zeiss Axiovert 200M).

244

245 **2.3.3.2. Murine bone marrow derived dendritic cells**

246 Bone marrow cells were obtained from femurs and tibias of C57BL/6 mice and treated with
247 red blood cells lysis buffer. All mice were raised in SPF environment with free access to standard
248 food and water. This study was approved by the Animal Ethics Committee of the University Claude
249 Bernard of Lyon. The bone marrow cells were differentiated into DCs as described previously
250 [Gosavi, 2018] by resuspension in RPMI-1640 culture medium containing 10% (v/v) fetal bovine
251 serum (Sigma, St-Quentin Fallavier, France), 1% Mycokill AB (GE healthcare, Pasching, Austria)
252 and 1% Penicillin / Streptomycin (Dutscher) with 20 ng/ml granulocyte-macrophage colony-
253 stimulating factor (GM-CSF) (Sigma, St-Quentin Fallavier, France)) and 50 µM 2-mercaptoethanol.

254 They were cultured for 5 days at 37°C with 5% CO₂ to ensure differentiation and obtain CD11c+
255 cells. DCs were seeded on Lab-Tek II Chamber Slides™ 4-well Glass slides overnight at 37°C with
256 5% CO₂. After 24 hrs, cells were incubated with NR-MPLA-NPs and CS-NR-MPLA-NPs at a 1:100
257 dilution for 24hrs, after which the medium was replaced. The cells were observed by fluorescent
258 microscopy (LEICA DM400 B LED).

259

260 **3. Results**

261

262 **3.1. Characterization of antigens**

263 The recombinants proteins EgA31 and EgTrp, previously studied and characterized by our
264 team (Esteves et al., 2003; Pétavy et al., 2008), were purified by Adriana Esteves' team. SDS-
265 PAGE and MALDI-TOF mass spectrometry are shown in figure 2 and supplementary data (S1),
266 respectively.

267

268 **3.2. Characterization of the vaccine nanoparticles**

269

270 Both blank and antigen-loaded MPLA-NPs were obtained with a mean size around 200 nm
271 (Table 1). All NP preparations showed a slight negative surface charge of about -3 mV. The
272 encapsulation efficiencies for the two antigens were near 85% (Table 1). Moreover, scanning
273 microscopy images confirmed the spherical shape and the measured size of the NPs (Fig. 1A, B
274 and C).

275 SDS-PAGE analysis was carried out on the antigen extracts from total NP suspension, the
276 supernatant and the pellet. The extraction method didn't alter the antigens' integrity since the
277 antigen had similar bands whether acetonitrile-treated or not (Fig. 1D and E). The molecular mass
278 of the antigens eluted from the NPs were consistent with their apparent molecular mass in the
279 *EgTrp* (27 kDa) and *EgA31* (54 kDa) controls, evidencing a lack of impact of the encapsulation
280 process on the integrity of the two encapsulated antigens (Fig. 1D and E). Accordingly, a high
281 percentage of the antigens was encapsulated into NPs without alteration of their integrity.

282 The capacity of the nanoparticle delivery system to protect the antigens' integrity in gastric and
283 intestinal condition was also assessed. The *EgTrp*-MPLA-NPs and *EgA31*-MPLA-NPs were
284 exposed to gastric (pH 1.6) and intestinal (pH 6.8) conditions for 2 and 6 hours and no alteration on
285 antigen integrity was observed (Fig 2 A and B). The test was done also in simulated gastric fluid
286 supplemented with pepsin and the antigen integrity was conserved (Fig 2C). These observations
287 indicate the potential of the nanoparticles in antigen protection against enzymatic degradation and
288 the suitability of such system for oral delivery of these antigens.

289 Different chitosan concentrations and incubation periods were tested regarding positive Zeta
290 potential to find out the optimal parameters for high CS coating. Figure 3A shows that incubation of
291 blank-MPLA-NPs with 0.1% CS for 4 hours was the most appropriate condition for surface
292 modification with a highest positive value (+ 30 mV) of the Zeta potential. Under these conditions,
293 CS-coated blank-MPLA-NPs displayed a positive surface charge until pH 7 with 8.6 mV measured
294 at pH 5.5 (Fig 3B) and kept their spherical shape as showed by transmission electron microscopy
295 (Fig 3C). The chitosan-coating of *EgTrp*-MPLA-NPs and *EgA31*-MPLA-NPs was then successfully
296 performed using the conditions selected above and the properties of these CS-MPLA-NPs
297 containing *EgTrp* and *EgA31* are shown in Table 2. Their positive surface charge and the
298 conserved antigen integrity are shown in Table 2 and Figures 3D and E. The encapsulation
299 efficiency was slightly decreased during this step because of NPs loss during the centrifugation
300 step.

301

302 **3.3. Elaboration and characterization of an oral vaccine form**

303 In order to confirm the oral vaccine efficiency, it was important to verify that NP size
304 distribution, positive surface charges of NPs and the integrity of the entrapped antigen were
305 maintained after freeze-drying and enteric capsule filling. As shown in figure 4A, freeze-drying did
306 not alter NP size since a similar size distribution was observed after reconstitution of the
307 lyophilizate in water. Different types of hard capsule were tested (gelatin and hypromellose/gellan
308 gum) and the gelatin-based capsules altered NP size distribution after the dissolution test, due to
309 their high moisture content not adapted for storage of the freeze-dried NP (data not shown).
310 Conversely, the hypromellose-based capsules containing gellan gum were more adapted and were

311 selected for their low moisture content and their delayed release that ensure long-term storage of
312 lyophilized NPs and an enhanced gastric resistance coating film, respectively.

313 Eudragit[®] L100-55-coated capsules displayed good gastric resistance at pH 1.2 for 2 hours
314 evidenced by disintegration and dissolution tests followed by rapid disintegration in 20 minutes at
315 pH 6.8. The NP size distribution was measured in dissolution medium showing that most of the
316 NPs' sizes were around 200 nm and that a small part of them formed 5 μ m aggregates (Fig. 4A).
317 Furthermore, the antigen kept its integrity and immunogenicity as shown by SDS-PAGE analysis
318 (Fig. 4B and 4C) and western blot analysis (Fig 4D) of NPs recovered from the dissolution medium.
319 Thus, a suitable oral form, consisting of enteric capsule filling by freeze-dried CS-*Eg*Trp-MPLA-
320 NPs and CS-*Eg*A31-MPLA-NPs, was obtained by preserving all NP characteristics to ensure an
321 efficient delivery of vaccine antigens to the target region of the dog's intestine.

322

323 **3.4. Effect of chitosan coating on NPs transport through Caco-2 cells**

324 The transport of NPs through intestinal epithelial cells was tracked using Caco-2 cell
325 monolayers and assessed by Nile red fluorescence tracking. Figure 5 shows the fluorescence of
326 the cells incubated with NR-MPLA-NPs and CS-NR-MPLA-NPs and illustrates the impact of
327 chitosan coating on NPs interaction with them. Without surface modification, NPs seemed to
328 accumulate in the intercellular region, with the red fluorescence mainly detected in extracellular
329 spaces with limited intracellular fluorescence (Fig. 5A). Conversely, a higher fluorescence was
330 observed after a 2 h of incubation with CS-MPLA-NPs that localized in intercellular and intracellular
331 regions (Fig 5B). Electron microscopy performed after 24-h incubation showed the presence of CS-
332 MPLA-NPs clusters attached to the cell membrane and in the cytoplasm (Fig 5C and D). These
333 observations indicate the intercellular transport of CS-MPLA-NPs across Caco-2 cells and the
334 suitability of surface modification for optimal antigen intestinal delivery.

335

336 **3.5. Effect of surface modification on NP uptake by antigen presenting cells**

337 The cytotoxicity of MPLA-NPs and CS-MPLA-NPs was evaluated on J774 macrophages after 24
338 hours of incubation showing good tolerance of these formulations (more than 95% of viability) for
339 all the dilutions tested. Uptake experiments using NR-MPLA-NPs and CS-NR-MPLA-NPs showed

340 that they were rapidly internalized inside macrophages during the first hour of incubation with
341 massive red fluorescence observed inside the cells (supplementary S2). After 1 h, red fluorescent
342 spots appeared indicating that NPs localized inside cell cytoplasm lysosomes (supplementary S2).
343 These observations were confirmed by confocal microscopy performed after the end of incubation
344 (Fig. 6A and B). The remarkable red fluorescent spots observed inside macrophages for the two
345 formulations were more numerous with CS-MPLA-NPs.

346 Similar observations were made with bone marrow-derived DCs (Fig. 6C and D), where
347 internalization of NPs was clearly increased after chitosan coating. These results suggest the
348 compatibility of MPLA and CS and the effectiveness of using them together to increase NP
349 internalization and hence antigen delivery to antigen presenting cells.

350

351 **4. Discussion**

352 The aim of the present work was to develop a new delivery system for the two recombinant
353 antigens *EgTrp* and *EgA31* that would ensure an oral vaccination of dogs against *Echinococcus*
354 *granulosus*. In a previous pilot study, recombinant *EgTrp* and *EgA31* were used in vaccination
355 trials using attenuated *Salmonella enterica* serovar *typhimurium* as a delivery system and a high
356 level of protection had been induced (Pétavy et al., 2008). However, the use and the administration
357 of genetically modified bacteria could be a drawback for environmental considerations as well as
358 for the development of infectious diseases (Karch & Burkhard, 2016). In this context, an alternative
359 vaccine delivery system should meet the following criteria: (i) to ensure good encapsulation and
360 protection of recombinant antigens, (ii) to enhance antigen delivery to antigen-presenting cells at
361 the canine Peyer's patches level, (iii) to induce a significant stimulation inducing a strong immune
362 response and counterbalancing tolerance and (iv) to be safe, biodegradable and easily
363 administrable to dogs.

364 For this goal, polymeric-based nanoparticles have been selected since they are widely used for the
365 development of new vaccines and particularly for oral vaccination and their advantage in antigen
366 protection, antigen delivery and immune system stimulation has been well demonstrated, (Zhao et
367 al., 2014). For this purpose, poly(D,L) lactic acid (PLA) was used to entrap the two recombinant
368 antigens as this biodegradable polymer was previously used for antigen-loaded nanoparticles

369 displaying interesting physicochemical properties for interaction with APCs (Tyler et al., 2016).
370 Monophosphoryl lipid A (MPLA) is a lipopolysaccharide derivative and a potent Toll-like receptor 4
371 (TLR-4) ligand that has been approved in the European Community for HBV and HPV vaccines
372 (Tritto et al., 2009). Its efficacy when included in polymeric nanoparticles was demonstrated with *in*
373 *vitro* and *in vivo* models and especially for mucosal and oral vaccination (Elamanchili et al., 2007;
374 Sarti et al., 2011). Moreover, stimulation effects of MPLA in polymeric NPs were observed on
375 canine macrophages by Guldner *et al.* (Guldner et al., 2016) and on canine dendritic cells even at
376 low loading ratio (0.025% MPLA/PLA), as demonstrated in our previous work (Milhau et al., 2020).
377 For this study, we used MPLA-NPs formulation to encapsulate *EgTrp* and *EgA31*. The MPLA was
378 added at a 0.1% loading ratio in order to ensure a good stimulation for the *in vivo* study and this
379 percentage could be reduced in accordance with our previous results (Milhau et al., 2020).

380 Characterization of antigen-loaded NPs proved that this delivery system was able to efficiently
381 encapsulate, conserve and protect these antigens. The obtained antigen-loaded NPs had a mean
382 size about 200 nm considering that M cells internalize particles less than 1 μm , as demonstrated
383 for the uptake of PLGA-NP by murine Peyer's patches (Shakweh et al., 2005). The high
384 encapsulation efficiency (around 85%) and antigen protection against gastric and intestinal
385 conditions (Fig 2) by nanoparticles evidence the suitability of this nanosystem for oral antigen
386 delivery. The observed protective capacity against enzymatic degradation was in accordance with
387 previously demonstrated results for insulin-loaded polymeric nanoparticle where polylactide-co-
388 glycolide (PLGA) nanoparticles induced more than 60% of conserved insulin after similar
389 incubation conditions (Jain et al., 2012).

390

391 The main goal of our developed nanoparticle-based vaccine was to ensure an efficient antigen
392 delivery to mucosal immune cells present in the dog duodenum and jejunum where the pH is
393 higher than 5.5 whatever the diet (Grayzel & Miller, 1928; HogenEsch & Felsburg, 1992). The
394 delivery system should be pH sensitive and have enough mucoadhesivity in these target parts to
395 be internalized by M cells and hence by APCs (des Rieux et al., 2006). The NPs' size and surface
396 charge are important parameters in the design of such a system.

397 To increase the intestinal exposure time and mucodiffusion of our NP system, chitosan, a natural
398 cationic polysaccharide obtained by chitin deacetylation widely applied for mucosal drug delivery
399 and vaccines (Xia et al., 2015), was selected to coat their surface. Chitosan is well known to
400 increase the adhesion and permeability of nanoparticle delivery systems at the intestinal level, thus
401 enhancing antigen delivery to APCs due to its positive charge and its ability to open the tight
402 junctions at the epithelial intestinal barrier allowing paracellular transport (Sonaje et al., 2012).
403 Chitosan is known to modulate immune response. Cole et al. (2018) studied the effect on Caco-2
404 cells of Chitosan-based NPs containing ovalbumin as antigen on cell viability, epithelial tight
405 junctions and transepithelial permeation. However, the capacity of this system to produce immune
406 responses in vivo after oral administration, was limited to local mucosal but not systemic immune
407 responses. A recent study by Shim et al. (2020) focused on the induction of the Th2 response by
408 Chitosan-based NPs containing Mdh (*Brucella abortus* malate deshydrogenase) as active ingredient
409 in order to evaluate the impact on internalization and immunogenicity on M cells, Caco-2 and Raji
410 B cells. They found that M cells expressed a significant increase in the production of TNF- α , IL-1 β
411 and IL-6 and transcellular transport in the samples incubated with the NPs- Chitosan-Mdh
412 compared to Mdh alone.

413 In this work, an optimized protocol was used to obtain positively charged CS-MPLA-NPs at a pH
414 higher than 5.5 (Fig 2A and B), promoting electrostatic interactions of CS-MPLA-NPs with the
415 mucosa and hence their adhesion and retention on the dog intestinal epithelium. NP
416 characterizations after chitosan coating confirmed the presence of a chitosan layer on their surface
417 in accordance with the results obtained for chitosan-coated PLGA NPs designed for intranasal
418 delivery regarding nanoparticle surface charge (Wang et al., 2013; Dou et al., 2019). Our protocol
419 was successfully applied on *EgTrp* and *EgA31*-loaded MPLA-NPs without noticeable antigen
420 alteration (Fig 3D and 3E).

421 To ensure efficient targeting to the dog duodenum and jejunum by antigen-loaded NPs, the pH
422 sensitivity of the delivery system is an important feature. In addition, it has to be easy to
423 administrate and stable during long storage. For these reasons, the developed NPs were freeze-
424 dried and inserted into hard capsules to ensure a good storage and to facilitate oral administration
425 to dogs. Eudragit[®] L100-55, an enteric coating polymer soluble above pH 5.5 was used to coat the

426 capsules, thus enhancing the gastric protection of our system and allowing to focus their activity to
427 the target region of the canine intestine where the pH is higher than 5.5. A similar approach for oral
428 vaccination has been developed for a *H. pylori* recombinant antigen by including hydroxypropyl
429 methylcellulose phthalate, a pH sensitive polymer, into PLGA-NPs and mice orally immunized with
430 this formulation showed a significant protection (Tan et al., 2017). However, including a pH-
431 sensitive polymer into the formulation of NPs was an interesting approach to protect the antigen
432 from degradation, but had a limited efficacy for targeting a specific area of the intestine. In the
433 present study, the antigens were protected against the degradation in the gastric and intestinal
434 tract by the enteric coating of the capsule and by the nano-encapsulation of the antigens. Our
435 results confirm that the antigen integrity was conserved in the simulated conditions (Fig 2).

436 The compatibility of MPLA and chitosan was verified on NP internalization by J774 macrophages
437 and Bone marrow-derived dendritic cells showing higher uptake profile in the case of CS-MPLA-
438 NPs (Fig 6). Moreover, the interaction with the intestinal epithelium could be promoted, since *in*
439 *vitro*, CS-MPLA-NPs preferentially accumulated in inter- and intracellular regions in Caco-2 cell
440 monolayers compared to uncoated nanoparticles (Fig 5). Our observations are in accordance with
441 the ones obtained with fluorescein isothiocyanate-labeled chitosan formulated into nanoparticles as
442 reported by Jia *et al.* (2009). They reported the transcellular transport of chitosan nanoparticles
443 through Caco-2 monolayers which was initiated by electrostatic force between the positive charged
444 nanoparticles and the negatively charged cell membrane. Similar results were reported by Dou *et*
445 *al.* (2019) for chitosan-coated PLGA nanoparticles and their interaction with Caco-2 monolayers
446 which showed that chitosan coating increases the particle attachment and then the internalization
447 by Caco-2 cells. These chitosan-coated PLGA nanoparticles showed a significantly improved
448 cellular uptake and transcellular transport and therefore exhibited a better transcellular permeability
449 of poor-soluble drugs. The enhancement of nanoparticle adhesion and retention at the mucosal
450 epithelium by chitosan coating allowing better antigen delivery and hence the induction of mucosal
451 immune response was highlighted by previous reports of nano-based mucosal vaccination. In the
452 case of a Hepatitis B surface antigen-loaded PLGA-NPs, the intranasal administration induced
453 higher level of serum IgG and secretory IgA for the chitosan-coated particles (Pawar et al., 2013).
454 In addition, Cole *et al.* (2018) showed that ovalbumin-loaded chitosan nanoparticles had an

455 enhanced permeability through Caco-2 monolayers triggering a higher mucosal IgA response
456 following oral immunization of mice.

457 Our developed oral vaccine in the form of freeze-dried CS-EgTrp MPLA-NPs and CS-EgA31-
458 MPLA-NPs, filled into enteric-coated capsules, was used for a preliminary *in vivo* study carried out
459 on a limited number of dogs and gave encouraging results (data not shown).

460 Oral vaccines against Echinococcosis and especially those based on nanoparticles as vectors are
461 very scarce. In addition, the majority of dog vaccination trials used injectable formulations, oral
462 vaccination trials have been carried out, but none have been conclusive so far. Gottstein *et al.*
463 developed a vaccine using the II/3-10 gene fragment from *Echinococcus multilocularis* coding for a
464 species-specific antigen expressed in live attenuated *Salmonella typhimurium* vaccine strain LT2
465 M1C (Gottstein *et al.*, 1990). Two vaccinated (subcutaneous and peroral) dogs showed a strong
466 humoral immune response to *S. typhimurium* antigens and to the recombinant *E. multilocularis*
467 antigen. Later Carol *et al.* (1998) demonstrated that nasal immunization using immunostimulating
468 complexes made from a *E. granulosus* tegumental antigen from protoscoleces induced secretory
469 IgA in the saliva and the serum of immunized dogs. In both reports, neither challenge infection was
470 carried out nor effect of the vaccination on the number of worms were described. Li *et al.* (2018)
471 tested the EG14-3-3 antigen loaded into MPLA-liposomes on *Echinococcus*-infected mice and
472 even if a systemic response with a high protective immunity was obtained after subcutaneously
473 immunization, the work remains limited to mice. Zhang *et al.* (2006) studied the protective effect of
474 3 injections of EgM4, EgM9 and EgM123 using Freund's adjuvant and their vaccine induced a high
475 level of protective immunity against *E. granulosus* infection in dogs, especially in terms of
476 suppression of worm growth and of egg development and embryogenesis. They later used Quil A
477 and ISCOM as an adjuvant to confirm the vaccine efficacy of the EgM proteins (Zhang *et al.*, 2018)
478 and also observed a systemic response with IgG production. These experiments show that the use
479 of adjuvants is not probably sufficient to change the immune response and the protective level.

480 Kouguchi *et al.* (2013) reported mucosal vaccination experiments against *E. multilocularis*. Dogs
481 were vaccinated using 500 µg of crude SRf1 antigens mixed with 1 cholera toxin (CT) subunit B
482 administered nasally followed by 3 boosters of SRf1 administered orally. They observed a great

483 reduction in the number of worms confirming the interest of mucosal immunization. Recently Vogt
484 *et al.* (2018) developed a method for oral immunization displaying heterologous proteins on the
485 surface of *B. subtilis* biofilms by introducing the coding sequences of *EgA31* and *EgTrp*. The
486 application of the recombinant *B. subtilis* spores led to the colonization of the gut and the induction
487 of specific IgG and IgA antibodies, but no challenge infection has been reported. More recently, in
488 2021 Pourceif *et al.* tested on dogs and sheep in a pilot study, a multi-antigen composed of EG95,
489 Eg14-3-3 and EgEnolase. The vaccine has been administered subcutaneously, the authors
490 assayed IgG and IgE antibodies and the cytokine IL-4 in the sera of immunized dogs and sheep.
491 The authors showed a significant protective effect in dogs but less significant in sheep.

492

493 **5. Conclusion**

494 In the present work we considered for the first time the use of a nanovaccine for mucosal
495 immunization. Surely, the vaccination of dogs could be effective for controlling Echinococcosis and
496 we think that our system, is a promising one. As for the *EgA31* and *EgTrp* antigens, their protective
497 effect have been shown and published previously (Fu *et al.*, 1999; Fraize *et al.*, 2005).

498 A nanoparticle delivery system encapsulating *E. granulosus* antigens was developed allowing the
499 successful activating effect on canine immunological cells. The addition of chitosan increased the
500 cell internalization of nanoparticles and promoted their adhesion to the mucosa. A preliminary *in*
501 *vivo* test on dogs gave a good protection rate and experiments will be performed in the future to
502 confirm the protective effect of our vaccine.

503 Our work answers some considerations defined as priority domains according to the One Health
504 strategy in so far as it deals with an important zoonosis disease. Hydatidosis, as mentioned in the
505 introduction to this article, includes different hosts with repercussions on human and animal health
506 and on the agricultural economy. Vaccination of dogs is a perfect way to control the disease and
507 stop its spread. Our nonvaccine also integrates environmental aspects, since the polymer used for
508 the formulation is biodegradable and has no impact either on dogs, nor on the environment.

509

510 **Funding**

511 This work was financed by the European Union (project number: PARAVAC FP7-KBBE-2010-4).
512 Uruguaysito Benavides, Imène Hellel-Bourtal and Cecilia Silvarrey were recipient of a grant from
513 the same project. Marine Leroux was financed from our own funds.

514 **Ethics**

515 This study was approved by the Animal Ethics Committee of the University Claude Bernard of
516 Lyon:

517 Ethics committee: CEIIA

518 Institution : SCAR

519 Approval number and date: DR2015-09

520 **Consent**

521 All participants provided written informed consent to publish this study.

522 **Conflicts of Interest**

523 All the authors declare that they have no known competing financial interests or personal
524 relationships that could have appeared to influence the work reported in this paper.

525 Marine Leroux, Uruguaysito Benavides, Imène Hellel-Bourtal, and Cecilia Silvarrey carried out the
526 experimental work and also the tables and figures

527 Nadège Milhau, Thierry Marchal and Philippe Lawton participated in the experimental work and in
528 the drafting of the manuscript

529 Samia Lahmar supervised the in vivo experimental part and also proofread the manuscript

530 Adriana Esteves, Anne-Françoise Pétavy, Stephanie Briançon, Sandrine Bourgeois, Eyad
531 Almouazen and Samira Azzouz-Maache supervised all the work, wrote and corrected the
532 manuscript.

533 And all authors reviewed the manuscript

534 **Tables and figures captions**

535

536 **Table 1** Characterization of antigen-loaded MPLA-NP prepared by the double emulsion technique.

537 **Table 2.** Properties of CS-MPLA-NPs containing *EgTrp* and *EgA31*

538

539 **Fig 1. Scanning electron microscopy observations and SDS-PAGE analysis of the prepared**
540 **nanoparticles:** (A) Blank-MPLA-NPs, (B) *EgTrp*-MPLA-NPs and (C) *EgA31*-MPLA-NPs. The
541 images show the spherical shape and homogenized size near to 200 nm for the three formulations.
542 SDS-PAGE analysis after Coomassie Blue staining of *EgTrp* (D) and *EgA31* (E) extracted from the
543 NPs. Lane 1: molecular weight markers, lane 2: antigen (positive control), lane 3: antigen treated
544 with acetonitrile (control of the extraction step), lane 4: total antigen-loaded NP suspension after
545 treatment with acetonitrile, lane 5: supernatant, lane 6: pellet of NPs after treatment with
546 acetonitrile.

547 **Fig 2. Protection of the *EgTrp*-NPs against gastric and intestinal degradation.** A) SDS-PAGE
548 analysis of *EgTrp* extracted from *EgTrp*-MPLA-NPs after incubation in water or buffer solution at
549 pH 1.6 (simulating gastric conditions) for 2 and 6 h. (B) SDS-PAGE analysis of *EgTrp* extracted
550 from *EgTrp*-MPLA-NPs after incubation in water or buffer solution at pH 6.8 (simulating intestinal
551 conditions) for 2 and 6 h. (C) *EgTrp*-MPLA-NPs incubated for 2 h with pepsin
552 supplemented simulated gastric fluid (SGF). NPs without treatment were used as control.

553 **Fig 3. Characterization of CS-MPLA-NPs.** (A) Zeta potential measurements of the CS-MPLA-NP
554 suspension after incubation with various concentrations of chitosan for various time intervals, (B)
555 Zeta potential measurements of CS-MPLA-NPs as function of the pH. NPs were previously
556 incubated with 0,1% of chitosan for 4 hrs. (C) Transmission electron microscopy of CS-MPA-NPs.
557 (D and E) Integrity of the encapsulated antigens before and after chitosan coating assessed by
558 SDS-PAGE analysis compared to non-encapsulated antigens. (D) CS-*EgTrp*-MPLA-NP and (E)
559 CS-*EgA31*-MPLA-NP. Lane 1: antigen, lane 2: antigen treated similarly to the NP extraction
560 method, lane 3: antigen extracted from the whole NP suspension before chitosan treatment, lane 4:
561 from the supernatant, lane 5: from the pellet, lane 6: the total after chitosan treatment, lane 7: the
562 corresponding supernatant, lane 8: the corresponding pellet.

563 **Fig 4. Antigen loaded CS-MPLA-NPs characterization after freeze-drying and capsule filling.**
564 (A) Size distribution of CS-MPLA-NPs after freeze-drying and redispersion in water (green) and
565 after capsule filling and dissolution in pH 6.8 (blue) compared to the CS-MPLA-NPs original

566 suspension (red). (B and C) SDS-PAGE analysis of protein extracted from CS-MPLA-NPs loaded
567 with *EgA31* (B) and *EgTrp* (C) after freeze-drying and capsule filling. Free antigens were used as
568 controls (lane 1). Proteins were extracted from lyophilized NP (lane 2) or from the total suspension
569 (lane 3), the supernatant (lane 4) and the pellet (lane 5) of capsule-filled NP after dissolution in
570 intestinal medium. (D) Western blot analysis of *EgTrp* extracted from CS-MPLA-NPs before (lane 1)
571 and after lyophilization (lane 2).

572 **Fig 5. Nanoparticles transport through Caco-2 cells.** Fluorescent microscopy images of Caco-
573 2 cells 2 h after incubation with NR-MPLA-NPs (A) and CS-NR-MPLA-NPs (B). Transmission
574 electron microscopy of Caco-2 cells after 24 h incubation with CS-MPLA-NPs (C and D).

575 **Fig 6. Nanoparticles uptake by antigen presenting cells.** (A,B): Confocal microscopy images of
576 J774 macrophages after incubation for 2 h with NR-MPLA-NPs (A) and CS-NR-MPLA-NPs (B).
577 The samples were counterstained with the Hoechst 33342 fluorescent dye (blue). (C,D):
578 fluorescence microscopy images of DCs after incubation for 24 h with NR-MPLA-NPs (C) and CS-
579 NR-MPLA-NPs (D).

580

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699

700 **Supporting information**

- 701 **S2 Video. CS-NR-MPLA-NPs internalization by J774 macrophages during 2h of incubation**
702 **monitored by Nikon Biostation IM**