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Research paper

# Microencapsulation of *Streptococcus equi* antigens in biodegradable microspheres and preliminary immunisation studies

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**Abstract**

*Streptococcus equi* subspecies *equi* is the causative agent of strangles, a bacterial infection of the respiratory tract of *equidae*. Current strategies to prevent strangles rely on antimicrobial therapy or immunisation with inactivated bacteria, *S. equi* bacterin, or M-like protein (SeM) extract. The aim of this work was to investigate whether immunisation with whole killed *S. equi* or a bacterial lysate entrapped in poly(lactide-*co*-glycolide) (PLGA) microspheres might induce protective immunity to mice. Animals were treated with a dose of antigen equivalent to 25 μg of SeM. For intranasal route animals were primed on days 1, 2 and 3 and were boosted on day 29. For intramuscular route, primary immunisation was carried out with a single injection on day 1 and animals were boosted on day 29. On day 43 animals were submitted to a challenge with a virulent strain of *S. equi*. Vaccination with antigen-containing microspheres induced higher serum antibody levels in mice treated by the intranasal route, whereas intramuscular immunisation did not reveal any difference between control and treatment groups. Microencapsulated antigens achieved to fully protect mice against experimental infection irrespective of the route of administration used. Following intranasal or intramuscular administration soluble antigen failed to protect mice against challenge. These studies indicate that PLGA microspheres are a potential carrier system for the delivery of *S. equi* antigens.


*Keywords:* Microspheres; Mucosal delivery: Poly(lactide-co-glycolide); Strangles; Vaccine

## 1. Introduction

Strangles is an important infectious disease affecting horses. It is caused by *Streptococcus equi* subspecies *equi*, a member of the Lancefield C group streptococci, and is characterised by an acute, febrile, suppurative, lymphadenitis [1]. Horses suffering from strangles have mucopurulent nasal discharge and abscesses, which may often burst and exude. Affected populations present high morbidity levels and infection may cause chronic illness or even death [2]. Strangles is very contagious, especially with foals, spreading easily from horse to horse and often leading to large outbreaks. It has been recommended that horses be included in regular vaccination programmes but the efficacy of most available parenterally administered vaccines has been disappointing. These include the use of *S. equi* M-like protein (SeM) rich extracts, which have not made major improvements in the control of the disease because of failure to produce mucosal antibodies [2]. In addition, these vaccines may have undesirable side effects such as adverse reactions at the site of injection while vaccinated horses may still develop clinical strangles.

Locally produced nasopharyngeal antibodies play an important role in the immune response of horses to protein antigens of *S. equi*, suggesting the nasal mucosa as a promising immunisation route [3–5]. Effective immunisation

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against *S. equi* may therefore depend on the successful induction of a mucosal immune response. Immunising via mucosal routes to stimulate the mucosal immune system offers several advantages over parenteral vaccination, such as improved efficacy, ease and economics of preparation and dosing, and reduced side effects [6]. An attenuated live *S. equi* nasal vaccine has been on the market since 1998 (Pinnacle IN®, Fort Dodge Laboratories, USA) but its use has raised important questions about its safety, due to reactions including nasal discharge, abscessation of lymph nodes and other sites, allergic reactions, systemic responses and purpura-like signs [7].

Protective local and systemic immunity may be generated by the use of biodegradable microspheres, which have shown enormous potential as antigen carriers. Publications have emphasized their adjuvant properties and a great effort has been put into their development [8,9]. They protect the antigen from proteolytic enzymes and have a greater ability to gain access to the mucosal-associated lymphoid tissue (MALT) when compared to free antigens thus providing a means of targeted vaccine delivery [10]. Microparticulate vaccines induce a mucosal response in lymphoid tissues regardless of the target site and the response is paralleled by the appearance of antibodies in secretions of glands distant from the site of immunisation. For immunisation purposes *S. equi* antigens have been associated to several vaccine adjuvants, such as aluminium hydroxide [11], Freund's complete adjuvant [12], monophosphoryl lipid A/trehalose 6,6′-dimycolate [13], Havlogen® [14], sucrose acetate isobutyrate [3], cholera toxin [4] and *Escherichia coli* heat-labile enterotoxin B subunit [5]. However, to our knowledge microencapsulation of *S. equi* antigens in biodegradable microspheres has not been described. Moreover, it has been demonstrated that the development of vaccine formulations based on whole killed cells or bacterial lysates has the potential to be a cost-efficient alternative to component vaccines [15,16], retaining the antigen intrinsic adjuvanticity that is usually lost during purification procedures. Therefore, the aim of this work was to investigate whether immunisation with whole killed *S. equi* cells or a bacterial lysate entrapped in poly(D,L-lactide-co-glycolide) (PLGA) microspheres might induce protective immunity to a strangles murine model. Studies include the microencapsulation of antigens and the preliminary results of intranasal and intramuscular immunisation of mice, which were then submitted to a final challenge with a virulent *S. equi* strain.

## 2. Materials and methods

### 2.1. Materials

*Streptococcus equi* subsp. *equi* (strain CF32) ATCC 53185 and *S. equi* subsp. *equi* (strain LEX) ATCC 53186 were a kind gift from Prof. J.F. Timoney (University of Kentucky, USA). Resomer® RG502 (PLGA 50:50, MW 12,000 Da) was obtained from Boehringer Ingelheim (Germany). Polyvinyl alcohol (PVA, MW 10,000 Da, 88% hydrolysed; Sigma–Aldrich, Spain), and ethyl acetate (Merck, Darmstadt, Germany) were also used.

### 2.2. Animals

Specific pathogen-free male BALB/c mice (25 g), purchased from Harlan Iberica (Barcelona, Spain), were used in immunisation experiments and were allowed free access to food and water during these experiments. These mice were kept in sterile housing conditions in cages provided with high efficiency particulate air filter-bearing caps. All mice were used at 6–8 weeks of age and all experiments were performed in compliance with European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

### 2.3. Antigen preparation

The antigens used in these experiments consisted of whole killed *S. equi* cells (WKSe) and a *S. equi* lysate (Se-lysate). Briefly, the WKSe was obtained upon harvesting a *S. equi* LEX culture and treating the bacteria with formaldehyde, whereas the Se-lysate was obtained upon cell disruption by high-pressure homogenisation at 900 Psi (Avestin Inc., Ottawa, Canada). Both types of antigens were freeze-dried before microencapsulation (Christ Alpha 1-4 freeze-drier, B Braun Biotech International, Germany).

### 2.4. Microsphere preparation and characterisation

Microspheres were prepared using a modification of a previously described double-emulsion (w/o/w) solvent extraction method [17]. Briefly, a specific amount of antigen was dissolved in 300 μl of water and emulsified in 3 ml of ethyl acetate containing 500 mg of PLGA, using high-speed homogenisation (16,000 rpm; Silverson SL 2, UK) for 30 s. The resulting emulsion was further emulsified in 6 ml of an aqueous PVA solution (1% w/v) using high-speed homogenisation (16,000 rpm/30 s). The double-emulsion thus formed was then diluted with 300 ml of a 0.3% (w/v) PVA aqueous solution and kept under magnetic stirring for 5 min. Finally, the extraction of the organic solvent was accomplished by adding an equal volume of an aqueous solution of isopropanol (2% v/v) and stirring during 30 min. Microspheres were isolated by centrifugation, washed with double-distilled water, frozen at −20 °C and lyophilised at 0.070 mb, using a Christ Alpha 1-4 freeze-drier (B Braun Biotech International, Germany). Particle size and morphology were studied using laser diffractometry (Coulter LS130, USA) and scanning electron microscopy (SEM; Jeol-JSM 5200, Tokyo, Japan), respectively. The encapsulation efficiency (EE) was calculated according to the relationship:

$$EE\ (\%) = amount\ of\ antigen\ encapsulated\ (mg) / initial\ amount\ of\ antigen\ (mg) \times 100$$

The amount of antigen incorporated, expressed as total protein (% w/w) per unit weight of microspheres, was measured upon digestion with 0.1 M NaOH using the bicinchoninic acid (BCA) protein assay (Pierce, USA). Protein integrity was assessed before encapsulation and after extraction from the microspheres using SDS–PAGE (12% gels) as described elsewhere [18].

### 2.5. Antigen release studies

In vitro release studies were carried out with the lysate-containing formulations, using the rotating-bottle method (Diffutest, Eurand, Italy). Eppendorf tubes were loaded with 10 mg microspheres dispersed in 1.5 ml of 0.2 M phosphate buffer, pH 7.4. At selected time intervals the solutions were centrifuged for 5 min at 8000*g* (Sigma 112 Model microcentrifuge, B Braun Biotech International, Germany) and 50 μl of the supernatant was then withdrawn. After each sample was collected, an equivalent volume of fresh buffer was added to reset the volume to 1.5 ml before returning it to the incubation chamber. Total protein content was determined using BCA protein assay and dissolution curves were determined from triplicate runs. Protein integrity was controlled by SDS–PAGE electrophoresis (12% gels) as described above.

### 2.6. Immunisation and challenge protocol

Male BALB/C mice ($n = 5$/group) were immunised with one unique dose of freeze-dried WKSe or Se-lysate, either free or microencapsulated, equivalent to 25 μg of SeM-like protein, as shown in Table 1. Vaccines were obtained by suspending the microspheres, or the WKSe, or dissolving the Se-lysate in a certain volume of isotonic phosphate buffered saline, pH 7.4 (PBS), in order to obtain the required concentration. This preparation was carried out just before administration. For nasal route animals were primed on days 1, 2 and 3 and were boosted on day 29, by introducing 10 μl of the appropriate preparation into each nostril by means of a micropipette. For intramuscular route primary immunisation was carried out with a single injection of 100 μl of the appropriate preparation on day 1, followed by booster on day 29. Blood was sampled from all animals by cardiac puncture on days 0, 28, and 42, and the serum was pooled by group for analysis of total anti-*S. equi* subsp.*equi* specific IgG using an ELISA technique. On day 43 animals were infected by intraperitoneal injection of 100 μl of a *S. equi* subsp. *equi* (strain CF32) suspension containing $4.59 \times 10^8$ CFU/ml [19]. During the experiment, according to the Directive 86/609/EEC, mice presenting signs of suffering (loss of weight and prostration) were sacrificed using a non-suffering method and considered dead for the experiments. Finally, on day 66 all surviving animals were ethically sacrificed.

Table 1
Vaccines, delivery routes, morbidity and survival of immunised mice upon experimental infection with the virulent *S. equi* strain (results on day 66; $n = 5$ *per* group)

| Group | Preparation | Route | Morbidity (%) | Survival (%) |
|---|---|---|---|---|
| 1 | WKSe-PLGA microspheres | Nasal | 20 | 100 |
| 2 | WKSe-PLGA microspheres | i.m. | 20 | 100 |
| 3 | Se-lysate-PLGA microspheres | Nasal | 20 | 100 |
| 4 | Se-lysate-PLGA microspheres | i.m. | 20 | 80 |
| 5 | Free WKSe | Nasal | 100 | 0 |
| 6 | Free WKSe | i.m. | 100 | 0 |
| 7 | Free Se-lysate | Nasal | 100 | 0 |
| 8 | Free Se-lysate | i.m. | 100 | 0 |

i.m. – intramuscular.

### 2.7. Measurement of the immune response

Antibody response to *S. equi* antigens was determined using an ELISA technique. *S. equi* antigens (0.1 μg/ml) in 0.05 M sodium carbonate–bicarbonate buffer, pH 9.6 were added to 96 well Maxisorp Nunc-Immuno Plate (Nunc, Denmark) and incubated overnight at 4 °C. The wells were washed three times with Tris-buffered saline, pH 8.0 (TBS; 10 mM Tris(hydroxymethyl)aminomethane and 0.25 M NaCl; Merck) containing 0.05% Tween 20 (Merck) and 0.1% bovine serum albumin (Sigma–Aldrich), and were incubated at 37 °C for 1.5 h with 200 μl of TBS containing 0.3% skim milk powder (Merck) and 0.05% Tween 20. Dilutions of serum from 1:2 in TBS were incubated in plates for 2 h at 37 °C. The total amount of bound immunoglobulin G (IgG) was estimated by the addition of a specific anti-mouse IgG-alkaline phosphatase conjugated antibody (Sigma–Aldrich). After 1.5 h incubation at 37 °C the enzyme substrate *p*-nitrophenyl phosphate (Sigma–Aldrich) was added. The reaction was stopped by the addition of 2 M NaOH and absorbance at 405 nm measured in a SpectraMax Plus microplate spectrophotometer reader (Molecular Devices, USA).

### 2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyse the data from the challenge test. A $p < 0.001$ was considered as a statistically significant difference.

## 3. Results

### 3.1. Microsphere characteristics

The biodegradable PLGA microspheres, either empty or containing *S. equi* antigens, present a spherical smooth surface under SEM (Fig. 1). Upon antigen incorporation the microsphere mean diameter increases from 1.3 μm (empty microparticles), to around 6.0 μm ($D_{50}$ 5.2 μm). Encapsulation efficiency was as high as 100% (w/w) for WKSe and 80% (w/w) for Se-lysate, which represents a total protein

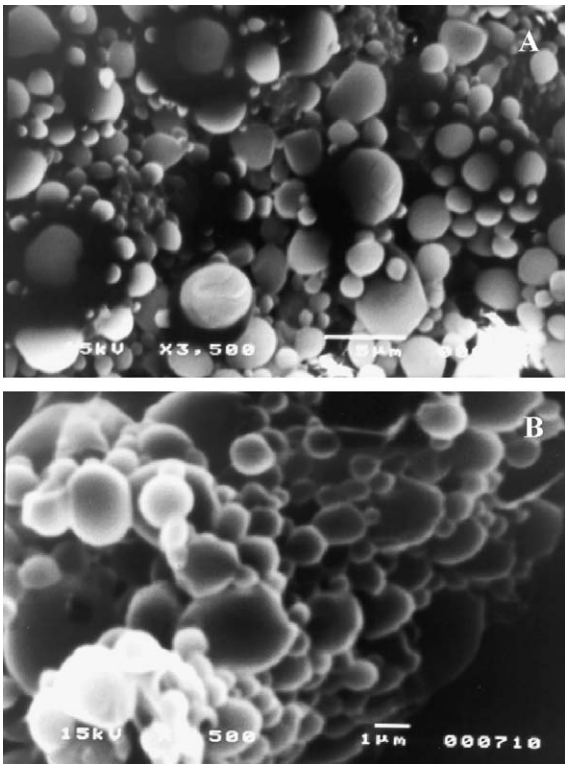


Fig. 1. SEM micrographs of PLGA microspheres: (A) microspheres containing whole killed *S. equi* cells; (B) microspheres containing a *S. equi* lysate.

content of 13.3% (w/w) and 10.9% (w/w), respectively. The SDS–PAGE analysis performed before encapsulation and after extraction from the microspheres to assess protein stability showed no changes in the pattern of protein migration suggesting that their integrity was maintained and therefore the antigen was unaltered throughout the microencapsulation procedure (Fig. 2).

### 3.2. In vitro release of the antigens

Fig. 3 shows the in vitro total *S. equi* protein cumulative release profiles from PLGA microspheres containing

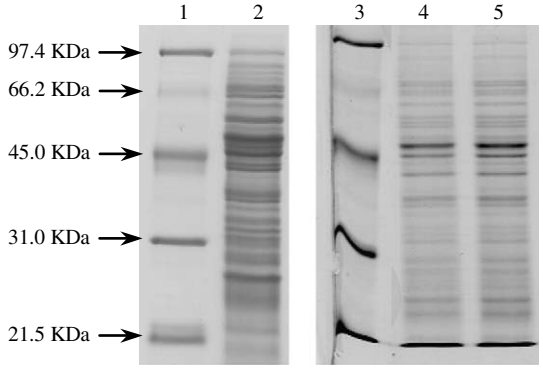


Fig. 2. SDS–PAGE (12% gel) of *S. equi* protein antigens. Lanes: (1 and 3) MW markers; (2) before microencapsulation; (4 and 5) after microencapsulation.

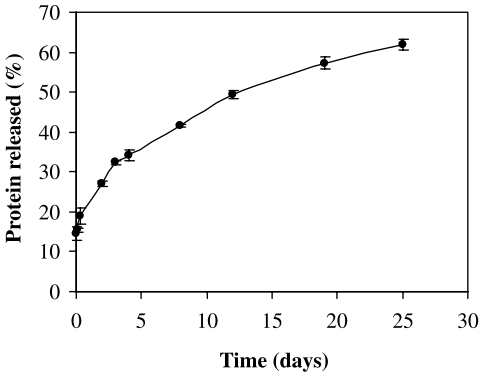


Fig. 3. In vitro release profile of proteins from enzymatic extract encapsulated in PLGA microspheres (mean ± SD; $n = 3$).

Se-lysate. After a burst release of 15% approximately, proteins are slowly released from microspheres, reaching a 60% cumulative release within 26 days.

### 3.3. Humoral serum immune responses

After intramuscular administration of WKSe or Se-lysate, neither the formulations nor the free antigens enhanced the immune response after primary immunisation. A strong increase in serum IgG titre was detected two weeks after booster, i.e. on day 42 (Fig. 4). However, there is no difference between the serum antibody titres elicited by microencapsulated antigens and those resulting from immunisation with the free antigens. The increase in systemic anti-*S. equi* IgG titre was almost immediate in the groups treated by the nasal route, although the immune response is not as strong as that elicited by the intramuscular route, even on day 42 (Fig. 5). Nevertheless there is a clear difference between the immune responses elicited by the intranasally delivered microencapsulated and free antigens. The primary immune response (day 28) is stronger for

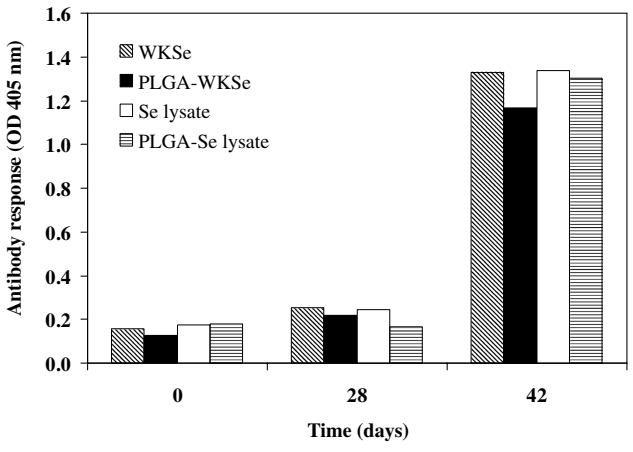


Fig. 4. Systemic humoral (IgG) response to intramuscularly delivered whole killed *S. equi* cells and *S. equi* lysate. BALB/c mice were immunised on day 1 and a booster was administered on day 29 (pooled samples; $n = 5$).

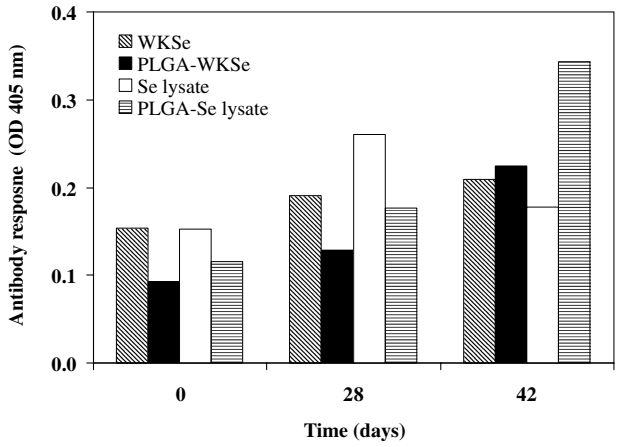


Fig. 5. Systemic humoral (IgG) response to intranasally delivered whole killed *S. equi* cells and *S. equi* lysate. BALB/c mice were immunised on days 1, 2 and 3, and a booster was administered on day 29 (pooled samples; $n = 5$).

the free antigens but on day 42, the titres produced by the microsphere preparations are higher than those obtained with the free antigens. In addition, immune responses to the microencapsulated antigens are still increasing, whereas those resulting from the free antigens either decreased (Se-lysate) or kept the same magnitude (WKSe).

### 3.4. Challenge with a virulent S. equi strain

Immunisation with antigen-containing PLGA microspheres achieved to fully protect mice against experimental infection with a virulent strain of *S. equi* (Table 1). Significant differences ($p < 0.001$) in morbidity and survival were found between animals immunised with microencapsulated and free antigens. Animals treated with WKSe or Se-lysate encapsulated in microspheres were protected against infection irrespective of the route of administration used. Morbidity signs that characterise strangles (e.g. loss of weight and prostration) were mild in some groups of animals treated with microencapsulated *S. equi* antigens although one animal died in group 4. On the other hand, following intranasal or intramuscular administration soluble antigens failed to protect mice against challenge. In these groups morbidity was severe, with external and internal abscess formation.

## 4. Discussion

The efforts put into the formulation of strangles vaccines have shown that the mode of antigen presentation to the immune system plays a crucial role on the efficacy of immunisation [4]. Successful vaccination against *S. equi* will involve intranasal antigen delivery as well as the use of an appropriate mucosal adjuvant. The efficacy of biodegradable microspheres as a vaccine adjuvant to induce systemic and local immune responses following mucosal immunisation has been extensively discussed and the adjuvanticity of most microparticulate formulations is considered to function on the principles of antigen protection from proteolytic enzymes, efficient phagocytosis and a better access to MALT, transport to the lymph nodes and prolonged antigen release [10,20].

In order to investigate *S. equi* antigen presentation, a soluble antigen (Se-lysate) and a naturally occurring microparticulate antigen (WKSe) were encapsulated in PLGA microspheres and the specific serum immune responses to these formulations were determined following intramuscular and intranasal delivery. The microspheres prepared present physicochemical properties that allow their use as a mucosal and parenteral vaccine delivery system [21], including a sustained protein release profile, thus originating a depot effect suitable for a prolonged stimulation of the immune system (Fig. 3). In addition, protein integrity is maintained throughout the micoencapsulation procedure, as demonstrated by electrophoresis (Fig. 2).

No difference was observed between the immune responses elicited by the several antigen formulations administered intramuscularly (Fig. 4). After priming specific serum IgG remained at levels similar to those found in non-immunised animals. The IgG titres increased only after booster indicating the effectiveness of the intramuscular route and its potential to induce a sustained immune response. However, the IgG titres elicited by intramuscular vaccination were not increased by antigen microencapsulation, probably due to the intrinsic adjuvanticity of some of the bacterial components as well as to the particulate nature of WKSe.

All preparations improved the systemic immune response upon intranasal immunisation. Microencapsulated antigens were able to produce stronger specific responses, particularly the Se-lysate. The free form of this antigen elicits a stronger primary IgG titre that decreases after booster, whereas the mice treated with microencapsulated Se-lysate presented an increasing response throughout the study. This observation is in line with the sustained protein release profile, which may be responsible for a long lasting immune response. In addition, microencapsulated antigens are taken up and processed differently, presenting different properties in terms of uptake and trafficking as compared with soluble proteins [20]. Even if only a small percentage of the nasally applied microspheres may translocate into the MALT and draining lymph nodes, particles can also access sites, such as the spleen, where they may induce systemic immune responses [20]. According to the geographical concept of immune reactivity, the antigen should reach the secondary lymphoid organs in an adequate concentration and during a sufficient period of time [22]. In mice treated intranasally, particle localisation in the respiratory tract is closely related to the vehicle volume, with higher volumes (50 μl) originating also microsphere deposition in the bronchopulmonary region from where they can translocate via the bronchus-associated lymphoid tissue (BALT) and reach the spleen, thus eliciting a systemic response [23]. In contrast, with small vehicle volumes

(10 μl) particle uptake and translocation is limited to the nasal-associated lymphoid tissue (NALT) eliciting comparable inferior responses in the spleen. In the present study we have used 20 μl, which may have reached both NALT and BALT, and therefore higher systemic IgG titres were obtained with the particulate antigens. Nevertheless, immune response increases slowly throughout the study, which may also be due to the volume used, according to the literature [23,24].

Interestingly, the increase in specific antibody titres in animals treated intranasally with free WKSe was also consistent throughout the experiment. The particulate nature of whole killed bacteria and the fact that bacterial antigens are exposed and ready to interact with the immune system may have played an important role. As a particulate antigen WKSe may be taken up by the MALT and induce a systemic response upon reaching the spleen. In contrast, the primary immune response to the encapsulated (i.e. non-exposed) WKSe increases only slightly. This may be due to the antigen slow release profile since IgG levels increase after booster when part of the antigen has been released by polymer erosion. Nevertheless, microencapsulation may reduce some undesirable effects that have been described for inactivated microorganisms, such as the potential toxicity of some of the bacterial components and inflammation at the site of injection [15].

Intranasal immunisation gave lower specific antibody titres than the same dose of *S. equi* antigens administered by the intramuscular route. This confirms our previous studies in which the relative efficacy of immunisation by nasal and intramuscular routes with tetanus toxoid encapsulated in poly(lactic acid) microspheres was compared [25]. In these studies the intramuscular route was the most effective one in increasing systemic immunity, even at a lower antigen dose. In fact, it has been reported that larger doses are required for nasal than for parenteral vaccines in order to achieve comparable systemic humoral responses [25,26]. Apart from the need for higher dosages, non-living nasal vaccines may also have to be given repeatedly in order to achieve adequate responses. Our results suggest that a higher vehicle volume and/or more intranasal administrations would probably further enhance systemic antibody levels. Bakke et al. [26] showed that intranasal immunisation stimulates immunological memory more rapidly in secretions than in serum and adequate systemic responses may be obtained by giving higher doses, at intervals longer than 4 weeks, in harmony with the intervals recommended for parenteral vaccines. According to these authors, mice may develop immunological memory of the mucosal as well as the systemic immune system after being immunised repeatedly by the intranasal route and antibodies in serum are markedly boosted by repeating administrations. However, this apparent disadvantage is overcome by the fact that intranasal immunisation also induces a significant mucosal immunity which is not obtained with intramuscular dosing [25]. This is particularly important for strangles, a disease of the upper respiratory tract of the equine populations where mucosal immunity is thought to play a crucial role [3,4], and is currently under investigation in our laboratories.

These studies clearly demonstrate that the encapsulation of *S. equi* antigens in PLGA microspheres, as well as their administration by the nasal and intramuscular routes, leads to an enhancement of specific immune response, causing full protection against the virulent strain (Table 1). Although all experimental animal groups showed increasing serum specific IgG levels, only those treated with antigen-containing PLGA microspheres were protected against experimental infection ($p < 0.001$), with very mild signs of morbidity. Therefore, antibody titres alone do not correlate with the protection found in this preliminary experiment. Nevertheless, there is a clear immunopotentiation effect of the PLGA microspheres, confirming the previous observations that antigen microencapsulation, or adsorption onto microparticulates confers a significant adjuvant effect [27,28]. The exact reason for this effect remains uncertain but, as aforementioned, microencapsulated antigens are taken up and processed differently, presenting different properties in terms of uptake and trafficking as compared with soluble proteins [20]. It is therefore of considerable interest to find out which immune cells are activated against *S. equi* when mice are immunised with microspheres to better understand the immune mechanism responsible for their strong activity upon microencapsulation. Both administration routes tested were equally effective against infection, which highlights the versatility of this approach. Protection may therefore be obtained through different combinations of administration routes and schedules of immunisation.

## 5. Conclusion

It was evident from this preliminary immunisation study that PLGA microspheres are a potential carrier system with adjuvant properties for the delivery of *S. equi* antigens. Microencapsulated *S. equi* lysate or whole killed cells given by the intranasal and intramuscular caused full protection against a virulent strain upon experimental infection. The immune response induced by these formulations may depend upon several factors acting simultaneously, rather than the action of a single parameter. The delivery of *S. equi* antigens should receive further attention in order to fully understand the mechanisms responsible for their strong activity upon association to PLGA microspheres.

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