In vivo and in vitro comparisons of spray-drying and solvent-evaporation preparation of microencapsulated *Mycoplasma hyopneumoniae* for use as an orally administered vaccine for pigs

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**Objective**—To evaluate the efficacy of an orally administered vaccine of *Mycoplasma hyopneumoniae* that was prepared by spray drying or solvent evaporation.

**Animals**—Thirty 6-week-old, crossbred, specific-pathogen-free (SPF) pigs.

**Procedure**—Pigs were randomly allocated into 5 groups and housed in an SPF facility. Pigs in 2 groups (groups AQ and CAP) were fed *M. hyopneumoniae* enteric-coated vaccine on days 0, 10, and 20. A third group (group IM) received an IM injection of *M. hyopneumoniae* vaccine with aluminium hydroxide as an adjuvant on days 0, 10, and 20. The last 2 groups (non-vaccinated-challenged [NV-C] and nonchallenged [NC]) were fed a sham treatment. All 24 pigs in groups AQ, CAP, IM, and NV-C were challenged exposed with 5 ml of a 10% pneumatic lung suspension administered on day 40 via intubation of the trachea. All pigs were slaughtered and the lungs removed and examined for lesions on day 68.

**Results**—In vitro studies indicated that these 2 microencapsulation techniques formed an effective shell and protected mycoplasmal antigen from gastric acid. Results of inoculation and challenge tests indicated that microencapsulated *M. hyopneumoniae* were sufficiently potent to induce an immune response and provide good protection.

**Conclusions and Clinical Relevance**—Orally administered microencapsulated *M. hyopneumoniae* vaccines induced an immune response and reduced the severity of lung lesions in challenged-exposed pigs. Results suggest that this novel method can be applied to other antigens, because the spray-drying process yielded an orally administered *M. hyopneumoniae* vaccine that induced a good immune response. (Am J Vet Res 2002; 63:1118–1123)

In swine, *Mycoplasma hyopneumoniae* is widely recognized as a potent pathogen that causes pneumonia by colonizing the respiratory tract epithelia and compromising epithelial integrity by inducing an inflammatory response. This pathogen also causes substantial economic losses by reducing weight gain and prolonging feeding periods. Certain drugs and antibiotics are effective in vitro against *M. hyopneumoniae* and can reduce clinical signs of the disease, but they have not yet been proven effective in eliminating the pathogen in vivo. Vaccination is considered to be an effective intervention strategy used to control pneumonia attributable to mycoplasma organisms, because *M. hyopneumoniae* infects ciliated epithelial cells of the respiratory tract, and a mucosal immune response may be elicited to prevent and control *M. hyopneumoniae*-induced pneumonia. The concept of a common mucosal immune system has been supported by several studies in which oral immunization was used. Oral administration of antigens can effectively induce antibodies through Peyer's patches. Pigs have some protection after IM inoculation of formalin-inactivated *M. hyopneumoniae* vaccine and a booster inoculation with the same vaccine onto the mucosal surface of Peyer's patches.

In another study by our laboratory group, we confirmed that an orally administered *M. hyopneumoniae* vaccine could protect pigs from infection with *M. hyopneumoniae*. The solvent systems used in the process to prepare the vaccine have potential risks regarding flammability and environmental pollution because they include organic solvents. Unfortunately, use of water as a solvent can result in problems of evaporation (ie, high expense to evaporate water and dry the compound), which would be an obstacle in mass production. Therefore, there is interest in alternatives such as the use of an aqueous coating. Ethylcellulose in aqueous polymeric dispersion is a formulated product that can be applied as a thin coating by use of a new method of film formation. This method of film formation involves coalescence of individual latex particles, which is distinct from that of water-soluble polymers or the organic solvent solutions of polymers. Less energy is required for removal of water during evaporation, the film is less porous, and transmission of water vapor through the film decreases after formation of the film. Spray-dried microparticles for drugs and antigens have been prepared by use of ethylcellulose aqueous polymeric dispersion, and this approach may provide a means for developing mycoplasmal vaccines.

**Materials and Methods**

**Animals**—Thirty 6-week-old specific-pathogen-free (SPF) crossbred pigs were purchased from an experimental farm. Pigs were allocated on the basis of weight into 5 groups...
(6 pigs/group). Pigs were housed throughout the study in an SPF facility and provided aseptic feed and water.

Preparation of microspheres—Mycoplasma hyopneumoniae strain PRIT-5 was cultured in Fries medium and treated as described elsewhere.\(^a\) Cultures were harvested when the optical density of the medium (measured at 550 nm) reached 0.07 to 0.09. Following addition of 0.2% formalin, cultures were incubated at 37°C (with shaking) for 1 hour. Cultures then were stored overnight at 4°C. A pH-dependent enteric coating consisting of cellulose acetate phthalate (CAP) and another enteric coating consisting of aqueous ethylcellulose polymer were used for preparation of microspheres. Meanwhile, solvent evaporation, as described elsewhere,\(^b\) was used to prepare the microspheres. Operating conditions in a homogenizer were controlled at 20°C, 400 rounds per minute (RPM), 0.4% of nonionic surfactant, and 32.4% CAP. The spray-drying procedure involved mixing 125 ml of a solution of M. hyopneumoniae, 47.5 ml of water, 75 ml of encapsulating material (aqueous ethylcellulose polymer), and 2.5 g of hydroxypropyl methylcellulose acetate (HPMCA) succinate (grade A5-HF). Dispersion of M. hyopneumoniae in water-based enteric-coating materials was accomplished by use of the heated chamber of a spray dryer. Droplets containing polymer and additives (ie, M. hyopneumoniae medium, water, and HPMCA) were pumped through a high-speed disk and then sprayed into the chamber along with warm air. Microspheres were dried and harvested by use of a cyclone current generated from the spray dryer. Disk speed was adjusted to 30,000 RPM, and inlet air temperature was held between 40 and 50°C for a few seconds before it was cooled to 35°C.

Encapsulation efficiency of microspheres—Microspheres (0.3 g) were dispersed in 1.2 ml of PBS solution (pH 7.4) overnight at 4°C by use of agitation. The solution was centrifuged (6,000 × g for 5 minutes), and supernatant was collected and mixed with 4 volumes of 100% acetone and 5% sodium dodecyl sulfate (final concentration).\(^c\) This solution was stored at −20°C for 1 hour and then centrifuged (8,000 × g for 30 minutes). The pellet was then dried by use of a vacuum pump and redissolved in 50 μl of PBS solution for use in a Coomassie protein assay to determine protein content; this assay involved the use of standard solutions of bovine serum albumin. Concentration of remaining protein in the microspheres was measured for determination of encapsulation efficiency, which was calculated by dividing the optical density of M. hyopneumoniae (after in vitro dissolution by the total mass of M. hyopneumoniae (before encapsulation)). Surface topography of the microspheres was examined by use of a scanning electron microscope.\(^d\)

In vitro dissolution—The pH environment of the gastrointestinal tract was simulated by use of a modified procedure\(^e\) intended to mimic the release of enteric-coated drugs. Dissolution of enteric-coated microspheres was determined at 37°C and for various pH. Meanwhile, release of M. hyopneumoniae microspheres was facilitated by use of a rotating paddle dissolution apparatus.\(^f\) Microspheres were incubated in 500 ml of acid buffer (1 N HCl containing 0.2% [wt:vol] NaCl; pH 1.2) that was rotated in a dissolution tester at 100 RPM. After 2 hours, pH was adjusted to 7.4 by addition of NaHCO\(_3\).\(^g\) At predetermined intervals, each sample was removed and centrifuged at 6,000 × g for 1 minute, and triplicate measurements of protein content in the supernatant were conducted by use of a protein microassay kit.\(^h\)

Experimental design—The first day of the study was designated day 0. Microspheres were suspended in 5% acetic acid solution and administered orally to 2 groups of pigs. Microspheres prepared with ethylcellulose polymer (group AQ) and microspheres prepared with CAP (group CAP) were administered via a tube inserted into the esophagus. Each pig in those groups was administered 3 × 10^9 color change units (CCU) of M. hyopneumoniae vaccine on day 0 as well as days 10 and 20. A third group (group IM) received IM injections of M. hyopneumoniae vaccine (aluminium hydroxide was used as an adjuvant) on days 0, 10, and 20. The remaining 2 groups (nonvaccinated-challenge exposed [group NV-C] and nonchallenged [group NC]) were orally administered a sham treatment. Food was restricted to all 24 pigs in groups AQ, CAP, IM, and NV-C on day 40, and each pig was challenged with 5 ml of a 10% homogenate suspension of M. hyopneumoniae containing 10^9 CCU of M. hyopneumoniae via a tube inserted in the trachea.

On days 0, 10, 20, and 30, samples of blood, feces, nasal secretions, and saliva were obtained from the pigs and stored overnight at 4°C. Feces and swab specimens of nasal secretions and saliva were collected, placed in 0.5 ml of washing buffer (PBS solution containing 5% [wt:vol] phenylmethanesulfonyl fluoride, 0.2% [wt:vol] trypsin soybean inhibitor, and 0.2% [wt:vol] gelatin), and then centrifuged at 6,000 × g for 1 minute. The supernatant (ie, washing buffer) was collected and stored at −20°C until analyzed.\(^i\) All pigs were slaughtered on day 68, and their lungs were removed and examined for lesions.\(^j\) The scoring system for recording the area of the consolidated lesions derived from the fact that, in pigs with enzootic pneumonia, such lesions occur almost entirely in the apical and cardiac lobes of the lungs, in the intermediate lobe, and in the leading edges of the diaphragmatic lobes. Ten points were allocated to each apical and cardiac lobe, 5 points to the intermediate lobe, and 5 points to each leading edge of the diaphragmatic lobes; thus, if all these areas were totally consolidated, the pneumonic score would be 55.\(^k\) Portions of the lungs were collected and used for isolation of M. hyopneumoniae and other bacteria.\(^l\)

Determination of titers—Titers against M. hyopneumoniae were determined by use of an ELISA, as described elsewhere.\(^m\) Each well of microtiter plates was coated with 100 μl of M. hyopneumoniae lysate solution at a concentration of 1 μg in 0.1M sodium carbonate-bicarbonate buffer (pH 9.0) and allowed to incubate overnight at 4°C. Fluid then was removed from each well by use of a suction device. Blocking of antigen on the plates was accomplished by addition of 100 μl of PBS solution containing 0.5% skim milk followed by incubation at 37°C for 30 minutes. Plates then were washed 5 times with PBS solution containing 0.05% Tween-20 (PBST). Phosphate-buffer milk (PBS solution containing 0.05% skim milk) was used as a diluent for all collected samples. For quantitative analysis of antibodies, a standard curve of ELISA values was obtained by use of dilutions of a swine immunoglobulin reference serum.\(^n\) Serial dilutions (6, 2, 0.6, 0.2, 0.06, 0.02, 0.006, and 0.002 μg of IgG/ml or 1.4, 0.48, 0.14, 0.048, 0.014, 0.0048, and 0.0015 μg of IgA/ml) were added to triplicate wells on the plates.\(^o\) Values of unknown samples were determined on the basis of results for the standard curves. An antibody value of 1 U/ml was defined as the absorbance value of 1 μg of immunoglobulin reference/ml, whereas an antibody value of 1 mU/ml was defined as the absorbance value of 1 ng of immunoglobulin reference/ml. Therefore, standard curves ranged from 0.06 to 6 units of IgG and from 0.014 to 1.4 units of IgA.

The initial dilution of serum and washing fluids for swab specimens was 1:200 and 1:10, respectively. Then, 100 μl of diluted sample was added to each well, followed by incubation at 37°C for 1.5 hours. Wells then were washed 5 times with PBST, and goat anti-pig IgG and IgA alkaline phosphatase (diluted 1:2,500 with conjugating buffer [0.05M Tris
HCl, 0.15M NaCl, 0.1mM EDTA, and 0.05% Tween 20]) was added to each well. Plates were incubated for an additional 1.5 hours. Washer substrate was then added, and after a brief incubation (30 minutes), the optical density was determined at a wavelength of 405 nm by use of an ELISA reader.1

Statistical analysis—Results were analyzed by use of an ANOVA. Significant differences among treatment groups and time (day of study) were determined. Significance was assigned for values of $P < 0.05$.

Results
Preparation and characteristics of microspheres—The CAP microspheres were prepared by use of solvent evaporation.11 The CAP microspheres ranged from 300 $\mu$m to 2 mm in diameter. Microspheres made with aqueous ethylcellulose polymer were easily produced without the use of organic solvent through the spray-drying process described by Liao et al.9 Variations in enteric-coating materials and additives influenced size and shape of the microspheres. Particle size of spray-dried microspheres was influenced by atomizer speed and microsphere composition, and microspheres made with aqueous ethylcellulose polymer typically varied from 5 to 20 $\mu$m in diameter.

Characteristics of the microspheres were determined for acidic (pH 1.2) and neutralized (pH 7.4) phases (Fig 1). Microspheres were placed in a simulated environment, and morphologic changes during release were monitored by use of scanning electron microscopy. Surface of the microspheres remained unchanged for 3 hours at pH 1.2, but the microspheres became roughened and ruptured at pH 7.4.

Encapsulation efficiency of microspheres—Microspheres containing M hyopneumoniae were prepared by use of CAP or aqueous ethylcellulose polymer as coating material. Analysis of microsphere preparation revealed that the encapsulation efficiency differed significantly between the 2 coating materials.

Protein concentration extracted from microspheres prepared with each of the coating materials was measured. The solvent-evaporation process was inefficient, with a protein loading efficiency of only 32%. The spray-drying method obtained the highest protein loading efficiency, reaching a value of 93.5%. Thus, encapsulation efficiency of microspheres prepared by the spray-drying process was approximately 3 times higher than that for the solvent-evaporation method.

In vitro dissolution—When microspheres were placed in an acidic solution (pH 1.2) similar to that found in the gastric environment, less than 10% of the protein was released after 2 hours of incubation at 37 C (Fig 2). However, in a simulated intestinal environment (pH 7.4), most of the M hyopneumoniae antigens in the microspheres were released between 0.5 and 4 hours after initiation of incubation. Although the percentage of protein released did not increase at pH 1.2, the amount of protein released from CAP microspheres and microspheres made with aqueous ethylcellulose polymer was 80 to 100% when the solution was increased to pH 7.4.

Figure 1—Scanning electron micrographs of microspheres prepared with enteric-coating made of cellulose acetate phthalate (A and B) and aqueous ethylcellulose polymer (C and D). Notice the effect of pH on the structure of microspheres incubated at pH 1.2 (A and C) and pH 7.4 (B and D) at 37 C for 15 minutes. Bars for A and B are 100 $\mu$m; bars for C and D are 1 $\mu$m.
Immune response and protective effect—Groups vaccinated orally by administration of microspheres had an increased humoral IgG response to *M. hyopneumoniae* that increased over time (Table 1). Only low amounts of *M. hyopneumoniae*-specific IgA were detected in serum, saliva, feces, and nasal secretions on days 0, 10, and 20 (data not shown). However, the amount of *M. hyopneumoniae*-specific IgA in serum and saliva from vaccinated groups on day 30 was significantly different from that of the NV-C and NC groups (Table 2).

Pigs in the NV-C group had clinical signs such as coughing and rough hair after challenge exposure. During necropsy, typical lung lesions were observed in pigs of group NV-C. All of the pigs from the 3 vaccinated groups had lower pneumonic scores, compared with scores for group NV-C. Mean of the pneumonic scores for pigs in groups CAP and AQ was significantly less than the mean scores for pigs in group NV-C. *Mycoplasma hyopneumoniae* was cultured from lung specimens of 20 of 24 challenge-exposed pigs, whereas other bacteria were not cultured from lung specimens of any pig (Table 3). Furthermore, challenge exposure of the SPF swine revealed that oral and IM administration of vaccine provided similar protection against the formation of lung lesions.

**Discussion**

Microspheres prepared by both processes elicited a similar immune response in pigs following inoculation with encapsulated *M. hyopneumoniae*. We believe this confirms that the spray-drying process is suitable for production of antigen-loaded microspheres prepared with enteric-coating materials. In addition, analysis of the efficiency of microspheres made with aqueous ethylcellulose polymer revealed that approximately 95% of the entrapped antigens were rapidly released within 1 hour after insertion in a neutral solution (pH 7.4). Scanning electron microscopy revealed that the initial burst of *M. hyopneumoniae* release was related to pH of the medium, suggesting that *M. hyopneumoniae* would remain intact in gastric acid and disintegrate in the intestines. To protect an orally administered vaccine antigen from potential destruction by acidic pH and gastric enzymes, it would be best to control gastric emptying time in addition to use of the microencapsulating process. Most particles are < 0.3 mm in diameter when they pass through the pylorus. It is difficult to achieve a constant rate of release throughout the gastrointestinal tract for small controlled-release dosage forms such as microspheres. This approach typically requires large doses of antigen because of inefficient stimulation of gastrointestinal-associated lymphoid tissue. In this regard, microspheres prepared by use of the spray-drying process were smaller than those prepared by use of the solvent-evaporation method.
Results of in vitro dissolution revealed that *M hyopneumoniae* release was pH-dependent and resembled results obtained for the rotating paddle method. Meanwhile, the prompt release of microspheres made with aqueous ethylcellulose polymer differed from that of polylactic-co-glycolic acid microspheres in which an initial burst is followed by subsequent antigen release. Differences between the release patterns of the 2 types of microspheres may also cause efficacy of the 2 types of microspheres to differ. However, the protection for antigen-associated microspheres following exposure to low pH indicated that the microencapsulated antigen provides effective protection against simulated gastric conditions. The pH value of the intestines was measured after the pigs had been slaughtered. The pH range was 1.4 to 3.7 in the duodenum and 4.6 to 7.8 in the ileum. This can explain the rupture of microspheres near the ileum where Peyer's patches are extensively located. This enhanced protection may partially explain the enhanced immune responses elicited by antigens in the intestines. Various factors, including protection of the antigen from low pH conditions, controlled antigen release, and effective attachment of antigen to the Peyer's patches, may help increase efficacy of this type of preparation of microspheres.

Magnitude of the response to *M hyopneumoniae* that was induced in pigs orally inoculated with vaccine was similar to the amount of specific antibody in pigs inoculated IM with doses of vaccine. Greater systemic and local immune responses were elicited for the vaccinated groups than for the NV-C group, indicating that microspheres prepared by use of the spray-drying or solvent-evaporation methods could be used as an orally administered vaccine to elicit a sufficient immune response against infection with *M hyopneumoniae*.

Because the primary goal of mucosal inoculation is to induce secreted antibodies at mucosal surfaces at the site of inoculation as well as at distant mucosal surfaces, serum IgA was measured. Amount of anti-*M hyopneumoniae* IgA measured in feces and nasal secretions was much less than the amount measured in saliva. This may have been attributable to the fact that enzymes contained in excreta digested the IgA. The higher amount of anti-*M hyopneumoniae* IgA detected in saliva suggests local production of anti-*M hyopneumoniae* IgA in the mucosa. It also could have been attributable to preferential transport of serum IgA to the site of inoculation as well as at distant mucosal surfaces.

Lung lesion scores of challenged-exposed groups were significantly lower in vaccinated groups of pigs than in pigs of group NV-C, indicating that orally administered microspheres or IM administration of vaccine could effectively protect the respiratory tract of pigs from *M hyopneumoniae*.

The mechanisms responsible for protection against *M hyopneumoniae* challenge after inoculation with encapsulated antigen are not completely understood. However, secretory IgA antibodies play a major role and move from the intestines to the lungs after oral administration of vaccine.6,10

The spray-drying method appears to be the more attractive of the 2 preparation processes and is superior to conventional microencapsulation techniques, because water dispersion of enteric-coating polymer and its additives is easy to use on a large scale. This method aerosolizes the mixtures to create microdroplets that solidify when subjected to a brief blast of heated air. From a manufacturing viewpoint, this technique also has the advantage of being a single-step process.23 Development of a new efficacious vaccine that is easy to administer would attract much attention. Such a vaccine should ideally possess consistent release and good bioavailability, and it should protect the antigen from acidic conditions. Microspheres can be formulated as tablets and mixed with the diet, which would protect *M hyopneumoniae* from the acidic environment and provide an easy means for mass administration of vaccines. In the study reported here, orally administered vaccine provided good immune responses and protective effects. Administration of microspheres as tablets in the diet would reduce most of the labor cost associated with IM administration of vaccines.

Finally, we concluded that *M hyopneumoniae* can serve as a model for encapsulation of other antigens with enteric-coating material. Use of the spray-drying method to prepare microspheres is convenient and reliable and should be considered for future use with other antigens.

References

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