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Development of an Immunomagnetic Method for Selective Isolation of *Actinobacillus pleuropneumoniae* Serotype 1 from Tonsils

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An immunomagnetic separation technique (IMS) for the selective isolation of *Actinobacillus pleuropneumoniae* serotype 1 was developed. Superparamagnetic polystyrene beads (immunomagnetic beads [IMBs]) were coated with purified rabbit immunoglobulin G specific for *A. pleuropneumoniae* serotype 1. The antibody concentration, the number of IMBs, the incubation time, and the temperature of incubation influenced the recovery of the target bacteria. The sensitivity of the IMS technique was 1,000-fold higher than that of direct culture. When tonsils from animals from infected herds were tested, significantly more positive tonsils were detected by the IMS technique (68%) than by the standard procedures (22%). The method represents an innovative and highly sensitive approach for the isolation of *A. pleuropneumoniae* from carrier animals.

Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, a highly contagious disease that causes important economic losses worldwide (22). Of the 12 NAD-dependent serotypes described, serotype 1 is the serotype most commonly isolated from animals with clinical cases of pleuropneumonia in North America (18). Early identification of subclinically infected herds is important for the control of the disease since carrier animals are the main source of contamination of immunologically naive herds (22, 25). To identify these herds, serology and bacteriological culture of samples from the upper respiratory tract have been used (27). Although serological testing has been helpful in the control of swine pleuropneumonia (2, 4, 6, 8, 21), it has some limitations. Infected pigs may be serologically negative (19, 27), and when positive, serological results may be observed in the absence of clinical signs or pathological lesions. In these cases, isolation of the organism becomes important for confirming the presence of infection. Carrier pigs harbor *A. pleuropneumoniae* in their nasal cavities and/or in their tonsils (11, 19). However, these sites are heavily colonized with several other bacterial species, making the isolation of *A. pleuropneumoniae* very difficult and time-consuming, even with the use of selective media (10, 27).

Immunomagnetic separation (IMS) methods allow for the specific recovery of target bacteria from highly heterogeneous suspensions (26). The method relies upon the interaction between cell-surface antigens and specific antibodies that are attached to magnetic polystyrene beads (24). This technique has been used to isolate pathogens such as *Escherichia coli* from bovine feces (3) and *Salmonella* spp. from blood and stool samples (14). The aim of this study was the development of an IMS technique for the selective isolation of *A. pleuropneumoniae* serotype 1 from the tonsils of carrier pigs.

The reference strain of *A. pleuropneumoniae* serotype 1 (Shope 4074, ATCC 27088) was used to standardize the IMS technique. *A. pleuropneumoniae* reference strains of other serotypes came from our own collection. Growth conditions on PPLO selective agar have already been described (27). For some parts of the study, a strain of *Pasteurella multocida* (strain ATCC 12948) was used and was grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.).

Production of rabbit polyclonal antibodies against strain Shope 4074 and the adsorption of the serum with reference strains of serotypes 2 through 8, 10, and 12 of *A. pleuropneumoniae* were carried out as described previously (17). Since the O-chain lipopolysaccharides of serotypes 1, 9, and 11 are antigenically similar (13), the serum was not adsorbed with serotypes 9 and 11 to avoid significant antibody titer reduction against serotype 1. The specificity of the serum for *A. pleuropneumoniae* serotype 1 as well as for serotypes 9 and 11 was confirmed by an indirect enzyme-linked immunosorbent assay (ELISA) (13). The immunoglobulin G (IgG) fraction was purified with a protein A chromatography column and was measured as described previously (16).

Superparamagnetic polystyrene beads (immunomagnetic beads [IMBs]) precoated with sheep anti-rabbit IgG (Dynabeads M-280; Dynal, Oslo, Norway) were used. The optimal concentration of *A. pleuropneumoniae* serotype 1-specific IgG antibodies to be used to coat the IMBs was determined with different concentrations of IgG incubated with 6×10^8 to 7×10^8 beads per ml for 3 h at room temperature on a shaker to avoid settling of the beads. Using a particle concentrator (MPC-M; Dynal), the beads were magnetized and retained on one side of the tube and were then washed twice in 1 ml of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) for 30 min each time with agitation at room temperature. The coated IMBs were then resuspended to obtain the original concentration (10 mg/ml) in 100 μl of PBS–0.1% BSA and were stored at 4°C. A volume of 20 μl of the different protein/bead ratios was added to 1 ml of 10^6 CFU of strain Shope 4074 per ml prepared in supplemented PPLO broth (Difco Laboratories) (27). An incubation of 30 min at room temperature with agitation was followed by two washes of 10 min each in PBS–0.05% Tween. The IMBs were plated

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TABLE 1. Effect of incubation time and temperature of incubation on the recovery of *A. pleuropneumoniae* serotype 1 from a mixed suspension with *P. multocida* by the IMS technique[a]

Time (min)	% Recovery at the following temp:	
	4°C	20°C
5	3.8 (1.9)	36.8 (4.1)
15	11.5 (6.9)	92.9 (19.5)
30	15.5 (3.6)	123.7 (24.6)
45	18.4 (3.3)	124.6 (26.9)
60	10.8 (0.6)	168.9 (34.7)

[a] Initial numbers of 10^3 CFU of *A. pleuropneumoniae* serotype 1 per ml and 10^6 CFU of *P. multocida* per ml. Results are expressed as mean (standard deviation).

onto PPLO selective agar. After 18 h of incubation at 37°C in 5% CO_2, viable counts were determined.

For each step, the mean of at least three independent assays is presented. The highest number of bound bacteria was obtained from a concentration of 5 μg of IgG per mg of beads, which is in agreement with previous reports (9, 15). No significant differences could be observed with higher concentrations of IgG. The number of bacteria recovered dropped drastically between 2.5 and 0.5 μg of IgG/mg of beads. In the absence of antibody, 0.01% of the bacteria adhered nonspecifically to the beads; similar nonspecific adherence has been reported elsewhere (1, 30). A concentration of 15 μg of IgG per mg of IMBs was chosen for the remainder of experiments to ensure an excess of antibody.

To evaluate the optimal coated IMB concentration, different numbers of coated IMBs (3×10^6, 6×10^6, 1×10^7, 2×10^7, and 5×10^7 beads) were added to 1 ml of 10^3 CFU of strain Shope 4074 per ml in PPLO broth. Incubation, washing, and plating were done as described above. Results showed that increasing the number of IMBs raised the number of *A. pleuropneumoniae* organisms recovered, from 29.1% with 3×10^6 IMBs to 81.8% with 1×10^7 IMBs. Increasing the number of IMBs to more than 10^7 did not seem to increase the number of bound bacteria. These results were similar to those obtained in other studies in which a low number of target cells was used (28, 29). In subsequent experiments, 10^7 coated IMBs were used.

Incubation time and incubation temperature may have some effects on the recovery of *A. pleuropneumoniae* and on the carryover of other organisms. To evaluate these effects, a mixed suspension of 10^3 CFU of strain Shope 4074 per ml and 10^6 CFU of *P. multocida* per ml was prepared in PPLO broth. A total of 10^7 IMBs were added to 1 ml of the mixture, and the mixture was incubated at 4°C and room temperature for 5, 15, 30, 45, and 60 min. The IMBs were then washed and resuspended in PBS as described above. Viable counts were determined in two different media: PPLO selective agar for the growth of *A. pleuropneumoniae* and *P. multocida* and BHI agar for the growth of *P. multocida* only.

As indicated in Table 1, the incubation temperature affected the recovery of *A. pleuropneumoniae*. When the incubation was carried out at 4°C, the rate of isolation of *A. pleuropneumoniae* was relatively low (below 20%). At room temperature (20°C), there was a considerable increase in the capacity of IMB to bind *A. pleuropneumoniae*, and starting from 30 min, a slight multiplication of the bacteria was found. Increasing the incubation period at room temperature from 5 to 60 min improved the recovery of *A. pleuropneumoniae*, which is in agreement with previous studies (28, 29). In all cases, the rate of recovery of *P. multocida* never exceeded 0.1%. An incubation time of 30 min at room temperature was chosen for subsequent experiments.

The IMS with artificially inoculated tonsils was evaluated as follows. Strain Shope 4074 was diluted to 10^3 CFU/ml in PPLO broth. Tonsils from high-health-status herds negative for *A. pleuropneumoniae* serotype 1 infection were used. Tonsils were cut into small pieces and added to the PPLO broth in order to obtain a final concentration of 0.1 g of tonsil/ml, and the mixture was vortex mixed. To evaluate the effect of the removal of cellular debris, supernatants were also filtered through filter paper (Whatman type 4; Whatman International Limited, Springfield Mill, Maidstone, England) before adding the IMBs. In parallel experiments, the PPLO broth was replaced by one of two other solutions which contained blocking agents: PBS–0.1% BSA or PBS–2% casein. A total of 10^7 IMBs were then added to 1 ml of the tonsil supernatant, and IMS was performed at room temperature for 30 min. Following performance of the washing procedures as described above, the IMBs were plated onto PPLO selective agar.

The ratio of *A. pleuropneumoniae*/normal flora obtained was significantly influenced by filtration of the tonsil supernatants and by using blocking agents. Before filtration and with the use of PPLO broth, the ratio was 1/1; after filtration, this ratio was improved to 7.4/1. The blocking agents PBS–2% casein and PBS–0.1% BSA were found to reduce the carryover, with ratios of 16.2/1 and 64.5/1, respectively. In contrast to other studies (5, 23), nonspecific adherence of bacteria was not reduced with the use of siliconized tubes or by changing the tubes between washings (data not shown). Thus, the optimal *A. pleuropneumoniae*/normal flora ratio was obtained when IMS was performed in PBS–0.1% BSA after filtration of tonsil supernatants.

To study the sensitivity of the IMS technique, a suspension of 10^4 CFU of strain Shope 4074 per ml was serial diluted in PBS–0.1% BSA. The count of each dilution was confirmed by a standard counting procedure on PPLO selective agar. Pieces of *A. pleuropneumoniae* serotype 1-negative tonsils were added to each dilution of the bacteria, and the mixture was then vortex mixed and filtered. Before adding 10^7 IMBs, each supernatant was plated onto PPLO selective agar (direct culture). The same supernatants were then used for IMS, which was performed by using the protocol of 30 min at room temperature. After washing, the IMBs were plated onto PPLO selective agar. Both tests were carried out by the same person. A plate was considered positive when a single colony of *A. pleuropneumoniae* serotype 1 was identified. Morphologically typical colonies of *A. pleuropneumoniae* were cultured onto PPLO agar and were identified as serotype 1 by testing for NAD dependence and urease reaction as described previously (27). Positive strains were serotyped as reported previously (18).

The sensitivity of the IMS technique was 1,000-fold higher than that of the standard procedure. Because of the overgrowth by other microorganisms on direct culture plates, no *A. pleuropneumoniae* counts could be done. The detection limits of *A. pleuropneumoniae* by direct culture and the IMS technique were 10^4 and 10^1 CFU/0.1 g of tonsils, respectively (Table 2). An increased rate of recovery of *A. pleuropneumoniae* was observed when a higher number of bacteria was present, from 32.2% (10^1 CFU/0.1 g of tonsils) to 91.7% (10^4 CFU/0.1 g of tonsils). In contrast to the work of Mortlock (20), no differences in the sensitivity of IMS from that of pure culture and from that with artificially inoculated samples were found (data not shown).

Finally, the validation of the IMS technique was achieved

TABLE 2. Sensitivity of the IMS technique obtained with tonsils artificially inoculated with *A. pleuropneumoniae* serotype 1 and comparison with direct culture

Initial no. of bacteria (CFU/ml)	*A. pleuropneumoniae* recovery	
	IMS	DC[a]
10^4	91.7 (8.4)[b]	+
10^3	67.9 (9.5)	−
10^2	57.3 (11.3)	−
10^1	32.2 (3.9)	−

[a] By direct culture (DC), overgrowth by other organisms precluded bacterial counts.
[b] Values are mean (standard deviation) percent recovery.

with a total of 150 tonsils from animals from three different herds confirmed to be infected with *A. pleuropneumoniae* serotype 1. Tonsils were randomly collected at the slaughterhouse and were separately stored at −20°C. Isolation of *A. pleuropneumoniae* serotype 1 from each tonsil was carried out by IMS and the standard procedure. Each tonsil was seared on the surface with a hot spatula. For the standard procedure, three parallel incisions were swabbed and the swabs were inoculated onto PPLO selective agar (27). For IMS, 0.3 g of tonsils was taken from an open cut and then reduced to small pieces with a scalpel and added to 3 ml of PBS–0.1% BSA. After vortex mixing and filtration of the supernatants, IMS was performed by using the protocol of 30 min at room temperature. IMBs were washed and plated onto PPLO selective agar. A count and a description of the types of colonies (natural flora included) on each plate were noted. Colonies suspected of being *A. pleuropneumoniae* were confirmed as being serotype 1 by a dot ELISA with two monoclonal antibodies directed against capsular epitopes (12, 13). Strains negative in tests with both monoclonal antibodies were tested for NAD dependence and urease production. Strains positive by both tests were serotyped (18).

Of the 150 tonsils from *A. pleuropneumoniae* serotype 1-infected herds, 19% were positive and 29% were negative by both IMS and the standard procedure. Forty-nine percent of the tonsils were positive by IMS alone and 3% were positive by the standard procedure alone (Table 3). The total percentage of *A. pleuropneumoniae* serotype 1-positive tonsils detected by IMS (68%) was significantly ($P < 0.01$; chi-square test) higher than that obtained by the standard procedure (22%). No other serotypes of *A. pleuropneumoniae* could be isolated by IMS, in comparison with the standard procedure, which allowed for the isolation of few strains of serotype 7 (data not shown). The number of *A. pleuropneumoniae* CFU/plate was higher by IMS

TABLE 3. Recovery of *A. pleuropneumoniae* serotype 1 from tonsils of animals from infected herds by the IMS technique and the standard procedure

Recovery by the following method:		No. (%) of tonsils[a]
IMS	SP[b]	
+	+	28 (19)
+	−	74 (49)
−	+	4 (3)
−	−	44 (29)

[a] A total of 102 (68%) of the tonsils were positive by the IMS technique. A total of 32 (22%) of the tonsils were positive by the standard procedure.
[b] SP, standard procedure.

TABLE 4. Distribution of *A. pleuropneumoniae* serotype 1 and normal flora recovered from infected herds by the IMS technique and the standard procedure

No. of CFU/plate	% Plated tonsils positive by the following method:			
	IMS		SP[a]	
	APP	Normal flora	APP	Normal flora
<10	26	36	75	3
10–300	58	60	19	22
>300	16	4	6	75

[a] SP, standard procedure.

than by the standard procedure, with 74 and 25% of the plates containing more than 10 CFU, respectively (Table 4). Also, the number of nonrelated microorganisms isolated by IMS was considerably reduced compared to that isolated by the standard procedure, with 4 and 75% of the plates having more than 300 CFU, respectively. For 7% of the samples positive by IMS, *A. pleuropneumoniae* serotype 1 was isolated in pure culture (data not shown). It has already been shown by standard methods that *A. pleuropneumoniae* is usually isolated only in low numbers per plate and is also easily overgrown by the contaminating flora (10).

One of the main concerns in porcine pleuropneumonia control programs is prevention of the entry of the organism into free herds through the introduction of carrier animals (27). Recently, a PCR for the detection of *A. pleuropneumoniae* in mixed bacterial culture of samples from tonsils has been standardized (7), with comparative recovery results similar to those obtained in this study. However, the PCR cannot differentiate among the different serotypes of *A. pleuropneumoniae*. Since most conventional herds are infected with different serotypes of *A. pleuropneumoniae* with low pathogenicities (27), this may lead to difficulty in interpreting a positive PCR result. The IMS technique described in this study is a sensitive, specific, and innovative method for the isolation of *A. pleuropneumoniae* serotype 1 from a heavily contaminated environment. The IMS technique can be adapted to the selective isolation of other important serotypes, such as serotypes 5 and 7, by changing only the specificity of the antibody used (unpublished data). Moreover, by this technique, viable bacteria are recovered, which may allow for antimicrobial sensitivity testing and the study of a larger number of *A. pleuropneumoniae* strains from subclinically infected herds. A better understanding of the epidemiology of this important swine pathogen may lead to the establishment of better surveillance programs.

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