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Comparison of Isolation Methods for the Recovery of *Bordetella bronchiseptica* and *Pasteurella multocida* from the Nasal Cavities of Piglets

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Nasal swabs from 241 piglets from 12 herds with clinical atrophic rhinitis and 283 piglets from 14 herds without clinical atrophic rhinitis were examined for the presence of *Bordetella bronchiseptica* and/or *Pasteurella multocida*. For *B. bronchiseptica*, swabs were streaked on three selective media. Blood agar supplemented with cephalexin was the most satisfactory selective culture medium for the isolation of *B. bronchiseptica*. For *P. multocida*, swabs were also streaked on three selective media. Mice were also used for isolation of *P. multocida* from the nasal cavities of pigs. The mouse inoculation test was not found to be the definitive test for the isolation of *P. multocida*. A significant number of *P. multocida* strains were avirulent in the mouse model. The modified Knight medium (without potassium tellurite) was the best single method for isolating *P. multocida*. However, a combination of mouse passage and direct culture on selective media increased the rate of isolation. There was no marked difference in the prevalence of *B. bronchiseptica* or *P. multocida* in swine herds with or without clinical atrophic rhinitis. Both capsular types A and D were present in the nasal cavities of the pigs with or without clinical atrophic rhinitis.

Bordetella bronchiseptica and *Pasteurella multocida* are well recognized as the etiological agents of atrophic rhinitis. Both *B. bronchiseptica* and *P. multocida* are widely prevalent in pig populations in countries with major swine-producing industries. However, the prevalence of these organisms greatly exceeds that of clinical atrophic rhinitis, as the organisms are found in herds both with and without the clinical disease (13). Both *B. bronchiseptica* and *P. multocida* grow on blood agar, but their isolation from field specimens is often complicated by the overgrowth of other organisms (25, 26); hence, more selective media are necessary for cultures. The roles of toxigenic *B. bronchiseptica* and/or *P. multocida* as the primary etiological microorganisms in atrophic rhinitis in swine have been well established by several workers (6, 11, 20, 21). Surprisingly, there are only a few reports on the utilization of selective media for the isolation of these microorganisms. Since the work of Farrington and Switzer (12), MacConkey furaltadone medium is commonly used for the recovery of *B. bronchiseptica*. Isolation of *P. multocida* in piglets has been done mainly by passage in mice (19). Recently selective media have been described (10, 26), but none of these has been evaluated in the field. The objective of this investigation was to compare isolation methods for the recovery of *B. bronchiseptica* and *P. multocida* from the nasal cavities of piglets from herds with and without clinical signs of atrophic rhinitis.

MATERIALS AND METHODS

Piglets. A total of 524 piglets from 26 pig farms with or without clinical signs of atrophic rhinitis were used in the studies. Four farms produced only weaner piglets with a 15-kg market weight, whereas all the other farms produced 100-kg market weight pigs.

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Collection of the nasal secretions. Nasal swabs (Calgi swab type 1 inolex) were used to collect the nasal mucus from 4- to 8-week-old piglets. Nasal swabs were introduced into the nasal cavity as far as possible and then placed in 2 ml of transport medium containing 50% fetal calf serum (Difco) in phosphate-buffered saline solution, pH 7.3 (Oxoid). The samples were kept on ice during the transport and examined by culture on the same day for the presence of *P. multocida* and *B. bronchiseptica*.

Isolation of *B. bronchiseptica*. Each swab was streaked onto three selective media: (i) MacConkey furaltadone medium without nystatin (12), (ii) Smith-and-Baskerville (S-B) medium without amphotericin B (Fungizone) (25), and (iii) blood agar medium supplemented with cephalexin.

Identification of *B. bronchiseptica*. All plates were incubated aerobically at 37°C for 48 h. Identification was carried out by standard methods (3). Gram staining; motility; tests for oxidase, catalase, and urease activities; reaction in triple sugar iron agar slants; and growth on MacConkey agar after subculture on blood agar were used as criteria to identify *B. bronchiseptica*.

Isolation of *P. multocida*. Two methods using a selective culture medium and/or mouse inoculation were used for the isolation of *P. multocida* from pig nasal swabs. Blood agar plates (tryptose soy agar [Difco] containing 5% bovine blood) were used initially for isolation of *P. multocida*. Later on, this medium was replaced by S-B medium (26) containing polymyxin B, bacitracin, and gentamicin (all three from Sigma), which was used for isolation of *P. multocida* from 122 nasal swabs. Selective Knight medium (15) containing clindamycin (Upjohn), gentamicin, and potassium tellurite was used for isolating *P. multocida* from 27 nasal swabs only. Modified Knight medium (without potassium tellurite) was used for isolating *P. multocida* from 202 nasal swabs.

Outbred 6-week-old male mice of strain CDI, weighing approximately 15 to 18 g were used for isolating *P. multocida* by the method reported by Pedersen and Barfod (19).

Transport medium (0.5 ml) containing nasal secretion was injected directly into one young adult Swiss albino mouse by the intraperitoneal route. The mice that died within 7 days of inoculation were autopsied and their livers were examined by culture for the presence of *P. multocida* on blood agar. The mouse inoculation method was used for isolating *P. multocida* from 482 pig nasal swabs.

Identification of *P. multocida*. The colonies of *P. multocida* were selected according to their morphological characteristics on different mediums. The isolates were subjected to a battery of different biochemical tests in order to characterize them in detail. The following tests were used: production of urease, nitrate reduction, decarboxylation of ornithine, and fermentation of various sugars such as arabinose, glucose, glycerol, lactose, maltose, mannose, sorbitol, trehalose, and xylose. The production of urease was determined on Christensen's medium (urea agar base; Difco). Decarboxylation of ornithine was evaluated by using Muller decarboxylase broth medium (Difco), and utilization of sugars was tested in phenol red broth medium (Difco). After inoculation, the media were incubated aerobically at 37°C. The readings were taken after 24 h for all the tests. A second reading was taken after 48 h only for the sugars which were negative after the first reading. Results were interpreted in accordance with the recommendations of MacFaddin (17).

Determination of capsular antigens of *P. multocida*. Capsular type A was identified by the hyaluronidase test (4), and capsular type D was identified by the acriflavine test (5). Isolates were also examined for their capsular types by indirect hemagglutination test (2). Antisera against reference strains of capsular types A, B, D, and E were produced in rabbits. Reference strains TS8 (type A), C81 (401/63) (type B), P.27 (type D), and Buniya II (type E) were obtained from G. R. Carter of the Regional College of Veterinary Medicine, Virginia-Maryland. Whole-cell–saline extract obtained after heating the whole-cell suspension at 56°C for 30 min was used for sensitization of human O group erythrocytes for the indirect hemagglutination test. The test was performed as described by Carter (2).

RESULTS

Identification of *B. bronchiseptica* and *P. multocida*. The isolates which were gram-negative motile small rods, able to grow on MacConkey agar and positive for citrate, oxidase, catalase, and urease, with no reaction at all in the butt of a triple sugar iron agar slant were identified as *B. bronchiseptica*. The isolates which were gram-negative nonmotile small rods, unable to grow on MacConkey agar and positive for oxidase, indole, glucose, mannitol, and sorbitol but negative for lactose, maltose, and urease were identified as *P. multocida*.

Comparison of selective media for the isolation of *B. bronchiseptica*. Nasal swabs from 120 piglets from herds with or without clinical signs of atrophic rhinitis were examined for the presence of *B. bronchiseptica*. *B. bronchiseptica* was isolated only from two piglets when S-B medium was used. However, removal of amphotericin B from this medium increased the number of isolates from 2 to 40. *B. bronchiseptica* was isolated from 46 piglets (38% positive) by using blood agar medium supplemented with cephalexin (data not shown).

Comparison between S-B culture medium and the mouse inoculation method for isolating *P. multocida*. Isolation of *P. multocida* from nasal swabs from 122 pigs was attempted by using S-B medium. *P. multocida* was isolated from 51% of the pigs by the combination of both methods. However, only 35% of the pigs were found to be positive for *P. multocida* by using S-B culture medium alone compared with 37% found to be positive by the mouse inoculation method (data not shown). Besides *P. multocida*, *B. bronchiseptica* also grew very well on S-B medium. However, it was difficult to differentiate these two bacteria simply on the basis of morphology, as colonies of both organisms were rose colored.

Comparison between selective Knight medium containing potassium tellurite and the mouse inoculation method for isolating *P. multocida*. Nasal swabs from 27 pigs from one herd were examined for the presence of *P. multocida* by using selective Knight medium containing potassium tellurite and the mouse inoculation method. *P. multocida* was isolated from 59% of the pigs by using both methods together. Only 26% of the pigs were positive by using selective Knight medium, whereas the mouse inoculation method yielded 54% of the isolates (data not shown). Preliminary studies demonstrated that selective Knight medium (with potassium tellurite) was inhibitory for the growth of type D strains of *P. multocida*, and thus, in subsequent studies it was replaced by a modified Knight medium, from which potassium tellurite was removed.

Comparison between modified Knight medium (without potassium tellurite) and the mouse inoculation method for isolating *P. multocida*. Isolation of *P. multocida* from nasal swabs from 202 pigs was attempted by using the modified Knight medium (without potassium tellurite). *P. multocida* was isolated from 54% of the pigs by using both methods together. Culture on modified Knight medium yielded 46% positive samples, whereas only 37% of the pigs were positive by the mouse inoculation method (data not shown).

The strains of *P. multocida* isolated by culture or the mouse inoculation method belonged to the same capsular type in all except four samples. In three of four samples, strains isolated by selective medium belonged to capsular type D, whereas by the mouse inoculation method the isolates belonged to type A. However, in the fourth sample, isolates belonging to type A were found when cultivated on selective medium and type D isolates were found by the mouse inoculation method. Strains belonging mainly to type D were isolated by using either the selective modified Knight medium (92%) or the mouse inoculation method (89%). However, the strains isolated by both methods together belonged to both type A (40%) and type D (60%) (data not shown).

The modified Knight medium also permitted the growth of *B. bronchiseptica*, and both of these bacteria were easily differentiated from each other by their colony characteristics.

Simultaneous comparison of three methods for isolating *P. multocida*. Three methods (modified Knight medium [without potassium tellurite], S-B culture medium, and mouse inoculation) were compared simultaneously for isolation of *P. multocida* from the same nasal swabs of 122 pigs. Generally, when *P. multocida* organisms were isolated on the S-B medium, they were also isolated on the modified Knight medium. Similarly, when the organisms were isolated on selective Knight medium containing potassium tellurite, they were also present on modified Knight medium. Of 122 nasal swabs tested, 107 were positive for *P. multocida* (88%) by using the modified Knight medium, whereas the mouse inoculation method yielded only 37% positive samples. Thus, the modified Knight medium proved to be the best single method for isolating *P. multocida* (Table 1).

TABLE 1. Comparison of the modified Knight and S-B culture media with mouse inoculation for isolating *P. multocida* from the nasal cavities of pigs[a]

Modified Knight medium	S-B medium	Mouse inoculation method[b]		
		+ (45)	− (77)	Total (122)
+	+	26	19	45
+	−	19	43	62
−	+	0	0	0
−	−	0	15	15

[a] +, positive; −, negative.
[b] Total numbers of swabs positive or negative are shown in parentheses.

Prevalence of *P. multocida* in swine herds in Quebec, Canada. There was no marked difference in the prevalence of *P. multocida* in swine herds with or without clinical signs of atrophic rhinitis. Forty-one percent of 524 nasal swabs examined were found to be positive for *P. multocida* on cultural examination. Both capsular antigen types A and D were isolated from pigs with or without clinical atrophic rhinitis. None of the isolates examined belonged to capsular antigen type B or E. The prevalence of isolation for individual farms was highly variable. The percentage of isolation varied from 0 to 100% of the piglets regardless of the clinical-atrophic-rhinitis status of the farm. Forty-seven percent of *P. multocida* isolates examined belonged to capsular type A, and 53% of the isolates belonged to type D (data not shown).

DISCUSSION

Although clinical signs of atrophic rhinitis are suggestive of infection, a definitive diagnosis is only possible by the bacteriologic examination of nasal secretions. *B. bronchiseptica* can be a significant nasal pathogen in young pigs, in which, acting alone, it is capable of inducing a mild to moderately severe degree of turbinate hypoplasia. The major significance of *B. bronchiseptica* infection in pig herds is the ability of the bacterium to initiate turbinate damage and assist the colonization of the nasal cavity by toxigenic strains of *P. multocida*. Although *B. bronchiseptica* is frequently isolated from young pigs in outbreaks of atrophic rhinitis, the infection also occurs widely in herds without this condition (13). Attempts to isolate *B. bronchiseptica* and *P. multocida* from the nasal cavities of pigs directly on blood agar plates were not always successful. A perusal of literature showed that various methods for isolating *B. bronchiseptica* and *P. multocida* have been described; however, no studies on the comparative evaluation of different methods for their isolation seem to have been carried out to date. Blood agar medium supplemented with cephalexin proved to be better than both McConkey and S-B media for isolating *B. bronchiseptica* from porcine nasal swabs.

Mouse inoculation seems to be the most commonly used method for the isolation of *P. multocida* from the nasal cavities of pigs. In the present investigation, the mouse inoculation method was used as a standard reference method for evaluating the effectiveness of three selective media for isolating *P. multocida*. The results shown in Table 1 indicate that the isolation rate of *P. multocida* was higher only when the modified Knight medium, compared with mouse inoculation, was used. There was also a close agreement between the isolation results of these two methods. The selective Knight medium containing potassium tellurite has been used successfully for the isolation of *P. multocida* from dogs and cats (15). However, the results obtained in the present study indicate that potassium tellurite in the Knight medium was inhibitory to the selective growth of *P. multocida* from pigs. Modified Knight medium (without potassium tellurite) resulted not only in the increased rate of isolation but also increased number of different strains of *P. multocida*. Thus, in cases in which only culture medium is used for isolation, the use of the modified Knight medium would be the best method for isolating *P. multocida* from the nasal cavities of pigs.

It is possible that mouse passage could favor selectively certain strains from the diverse population of *P. multocida* present in the nasal cavities of pigs. However, results of the isolation of capsular types by using mouse inoculation and modified Knight medium did not indicate any selective isolation of one type over others by either method. Hoffman et al. (14) compared Columbia blood agar supplemented with neomycin and bacitracin and the mouse inoculation method for effectiveness in recovering *P. multocida* from nasal swabs from pigs which had experienced enzootic or progressive atrophic rhinitis. They reported that the mouse inoculation technique was most effective in recovery of *P. multocida*. The results of our investigation clearly indicate that the mouse inoculation test is not the definitive method for isolation of *P. multocida*. The data presented imply that a significant number of *P. multocida* organisms did not kill mice. Curtis et al. (8) reported considerable variation in the virulence of *P. multocida* isolated from avian sources for mice. They further suggested the use of mice for screening *P. multocida* isolated from farm livestock for virulence, especially when failure to distinguish between avirulent and virulent strains of *P. multocida* may confuse the diagnosis.

Atrophic rhinitis is a serious, widely prevalent, contagious disease of swine. The disease is now divided into a nonprogressive form caused by toxigenic *B. bronchiseptica* and a progressive form caused by toxigenic *P. multocida* alone or in combination with *B. bronchiseptica* or other agents (9). The epidemiology of *B. bronchiseptica* and *P. multocida* infections in pigs is less well understood. Both organisms have been isolated from most domestic animal species, and because they are ubiquitous pathogens, there is always the risk that infection could be introduced by nonporcine vectors.

Results of this investigation did not show any qualitative difference in the population of *P. multocida* present in swine herds with or without any clinical atrophic rhinitis. These results differ from those of a majority of similar studies in which the prevalence of *P. multocida* was reported to be much higher in swine herds with atrophic rhinitis than in herds without atrophic rhinitis (18, 23, 24). This difference could be explained in two possible ways. Firstly, the earlier workers used neither a combination of isolation methods nor any modified selective media for isolating *P. multocida* from pigs as used in this study. Thus, the use of more sensitive methods permitted the isolation even from mildly infected pigs. The other possibility is the difference between pig rearing in Quebec and the pig-rearing methods used in several other countries. There is practically no existence of specific-pathogen-free herds in Quebec, and exchange of animals between different swine herds is a well-known fact. It is possible that all the swine herds in Quebec are infected with *P. multocida* but the disease may be expressed only in some herds. Characterization of the strains on the basis of their capsular antigens did not permit us to demonstrate any association with clinical atrophic rhinitis. These results are

similar to those reported by Sawata et al. (22). Bechmann and Schoss (1) examined tonsillar and nasal swabs from 468 piglets of about 8 weeks of age in 30 pedigree herds without any evidence of clinical atrophic rhinitis for the presence of *P. multocida*. *P. multocida* was detected in 27.4% of the tonsillar swabs and 18% of the nasal swabs. Most of the isolates belonged to capsular type D. Cowart et al. (7) reported that *B. bronchiseptica* and *P. multocida* were isolated more frequently from nasal swabs and lungs of 8-week-old pigs with higher atrophic rhinitis scores. Larivière et al. (16) reported that capsular antigen types A and D were both present in the nasal cavities of pigs with or without atrophic rhinitis. However, toxigenic isolates were found only in pigs with clinical atrophic rhinitis. Since both *B. bronchiseptica* and *P. multocida* are widely prevalent in the pig population, their total exclusion from a herd is possible only by the development of herds with high health status or a specific-pathogen-free system and strict maintenance of an effective barrier.

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