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# A SELECTIVE MEDIUM FOR THE ISOLATION OF P. MULTOCIDA IN NASAL SWABS FROM PIGLETS

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*Selective nutritive media and a mouse inoculation test were used for isolating P. multocida from piglet nasal swabs, taken from three herds with clinical atrophic rhinitis,. During the investigation a total of 149 nasal swabs was examined. Each swab was streaked on to 6 different selective media, which contained different combinations of chemotherapeutics: bacitracin, gentamicin, polymyxin B and clindamycin. The selectivity of nutritive media was conditioned by the content and concentration of chemotherapeutics. The highest percentage of positive findings (18,42%) was obtained on Columbia blood agar 2, when it contained bacitracin, gentamicin and clindamycin. The frequency of isolation of P. multocida, for different herds, varied from 7.14 to 34.28%. Applying the acriflavine test we found that 61.90% of the isolated strains of P. multocida belonged to type D. In the mouse inoculation test P. multocida was isolated in a considerably lower percentage (17.14%) in comparison to the selective media, blood agar 2 (28.57%) and Columbia blood agar 2 (34.28%).*

*Key words: P.multocida, selective media, pig, nasal swab.*

## INTRODUCTION

Progressive atrophic rhinitis (PAR) is a serious infectious disease of swine that reduces weight gain and causes atrophy of the nasal turbinate bones, sneezing, nasal haemorrhage and, in severe cases, twisting and shortening of the snout. The term Progressive Atrophic Rhinitis (PAR) was proposed for the infection with toxigenic Pasteurella multocida (capsular serotype D and A strains; De Jong, 1992.). In swine, P. multocida is sometimes associated with pneumonia but its major importance is in atrophic rhinitis (Giles et al., 1980.; Pedersen and Elling, 1984.). A protein-dermonecrotic toxin, produced by types A and D toxigenic strains of P. multocida, and concurrent infection with B. bronchiseptica appear to be major factors in the development of atrophic rhinitis. Bordetella bronchiseptica dermonecrotoxin produces nasal turbinate atrophy in piglets only up to a few weeks old, while P. multocida dermonecrotoxin acts up to several months of age

(De Jong at al., 1980.; Pedersen and Elling 1984.). It is recognised that toxin producing strains of P. multocida cause progressive and irreversible turbinate lesions in pigs previously infected with B. bronchiseptica. The etiological definition of Progressive Atrophic Rhinitis (PAR) implies that the ultimate diagnosis depends on the demonstration of toxigenic P. multocida in a herd (Bechmann and Schoss, 1988.; Schoss et al., 1985.). The prevalence of infection of toxigenic P. multocida serotype D was higher in herds with clinical disease but may be present in about 5% of the pigs in a herd with no clinical history of atrophic rhinitis (De Jong 1992.). In contrast to this report, Lariviere et al. (1993.) found no marked difference in the prevalence of P. multocida in swine herds with or without clinical atrophic rhinitis.

Toxigenic P. multocida grows readily in the laboratory but is difficult to isolate from nasal swabs because the bacteria are frequently overgrown by commensal flora. Selective media containing antimicrobial agents have been developed to promote the isolation of P. multocida from nasal swabs (De Jong et al., 1985.; Smith and Baskerville, 1983.). Isolation of P. multocida in piglets has been done mainly by passage in mice (Lariviere et al., 1993.). Bacitracin is used to suppress the accompanying flora (De Jong, 1992.) for isolation of P. multocida from the nasal chamber. Giles et.al. (1980), however, encountered considerable incidental contamination using bacitracin in blood agar. One medium 8HPG agar had particular promise for isolating pasteurellae selectively as easily recognisable colonies from the nasal flora (Smith and Baskerville, 1983.). The objective of this investigation was to evaluate different selective media for the recovery of P. multocida from the nasal cavities of piglets from herds with and without clinical signs of atrophic rhinitis.

## MATERIAL AND METHOD

Piglets: a total of 149 piglets from 3 pig farms with clinical atrophic rhinitis was used in the studies. Nasal swabs from 114 piglets were streaked on selective culture media. For comparison between the methods, selective culture media and the mouse inoculation method, were used for the isolation of P. multocida from 35 pig nasal swabs.

Collection of the nasal secretions: Nasal swabs were collected from 6-8 week-old piglets. Nasal swabs were placed in 2 ml of transport medium containing 5% calf serum in phosphate-buffered saline solution (pH 7.2). The samples were examined by culture on the same day for the presence of P. multocida.

Isolation of P. multocida: two methods using a selective culture medium and mouse inoculation were used for the isolation of P. multocida from pig nasal swabs.

Media: commercially available ingredients for media were prepared according to the manufacturers instructions. Additives were incorporated when the molten agar was about 50 $^0$C. Solutions of antimicrobial agents were freshly prepared and sterilised by filtration. Media prepared in such a way were kept at 4 $^0$C. Media were used at the latest 5 days after preparation.

P. multocida was cultivated on different solid culture media: blood agar (BA), nutrient agar (NA) and Columbia blood agar (CBA). Blood agar was constituted of tryptose soy agar base and 7% defibrinated bovine blood. Columbia blood agar was Columbia agar with 7% bovine blood.

Nutrient agar: nutrient broth 20 g, 2 g yeast extract, 75 mg ferric citrate, l0 g lactose, 24 mg phenol red and 18 g agar in one litre of water.

The nutritive media contained antibiotics and chemotherapeutics in the following combinations:

Blood agar 1. bacitracin 2,5 IU
2. bacitracin 2,5 IU + gentamicin 0,05 μg

Nutrient agar 1. bacitracin 2,5 IU + polymyxin B sulphate 0,20 μg
2. bacitracin 4 IU + gentamicin 0,03μg

Columbia blood agar 1.bacitracin 5 IU
2. bacitracin 2,5 IU + gentamicin 0,03 μg +clindamycin 0,2 μg

The above mentioned quantities of chemotherapeutics were added to I ml of media.

Mouse inoculation method: transport medium (0,5 ml) containing nasal secretion was injected into mice weighing ca 20 g 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. This method was used for isolating P. multocida from 35 pig nasal swabs.

Nasal swabs were streaked on different nutritive media and incubated aerobically at 37 $^0$C for 48 hours. Identification was carried out by standard methods (Carter and Subronto, 1973.).

Identification of P. multocida: The colonies of P. multocida were selected according to their morphological characteristics on different media.

The isolates were subjected to a series of different biochemical tests. The following tests were used: oxidase, catalase, production of urease, nitrate reduction and fermentation of various sugars such as glucose, arabinose, lactose, maltose, mannose, sorbitol, trehalose and xylose. After inoculation, the media were incubated aerobically at 37 $^0$C and readings taken after 24$^h$ for all the tests. For sugars, which were negative after the first reading, the incubation was prolonged up to 48 hours.

Identification of type D strains of P. multocida: Capsular type D was identified by the acriflavine test, (Carter and Subronto, 1973.). Strains of P. multocida were inoculated on brain heart infusion broth and incubated at 37 $^0$C for 16-24 hours. The bacteria were concentrated by centrifugation. A 1:1000 aqueous solution of neutral acriflavine (0,5 ml) was added to 0,5 ml of broth containing the bacteria. After mixing to resuspend the bacteria, the tube was left stationary at room temperature. If the strain being examined was type D, a heavy precipitate became evident within 5 minutes. After 30 minutes, the heavy precipitate settled, leaving a distinct supernatant.

## RESULTS

During our investigation a total of 149 nasal swabs was examined. Each martial was streaked on 6 different media containing different combinations of chemotherapeutics. The isolates which were gram-negative, nonmotile small

rods, unable to grow on MacConkey agar and positive for oxidase, catalase, glucose, mannitol and sorbitol but negative for lactose, maltose, urease were identified as P. multocida. After 24 hours of incubation on blood agar, colonies of P. multocida were smooth, gray-white, nonhemolytic, about 1-2 mm in diameter (Fig. 1), with a characteristic musty odour. There were no differences noted either in the growth or in the appearance of colonies on Columbia blood agar (Fig. 2). After incubation for 48 hours at 37 $^{o}$C colonies of P. multocida on NA medium were about two-thirds the size of those on blood agar, and pale greyish-pink (Fig. 3 and 4).

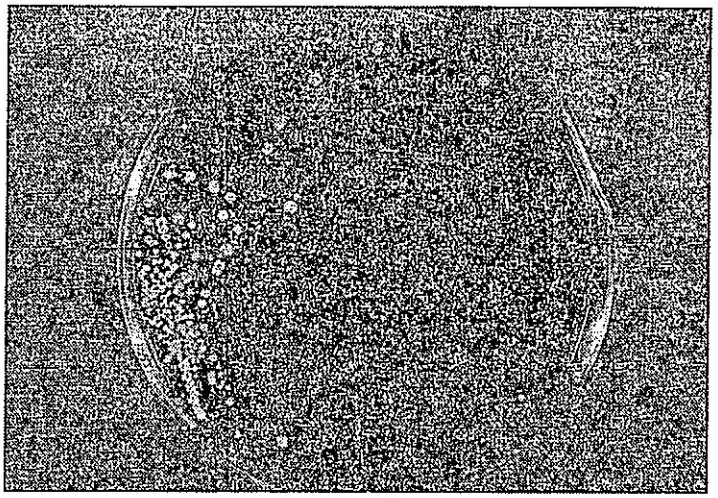

Fig. 1. P.multocida - 24$^{h}$ culture on blood agar 2

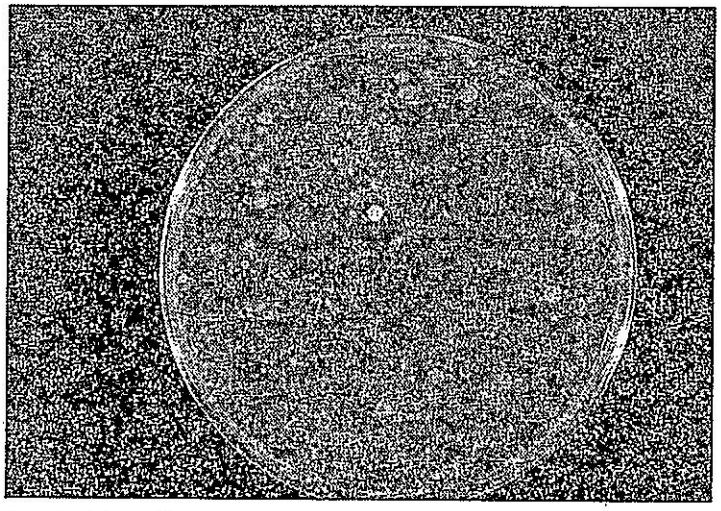

Fig. 2. P.multocida - 48$^{h}$ culture on Columbia blood agar 2

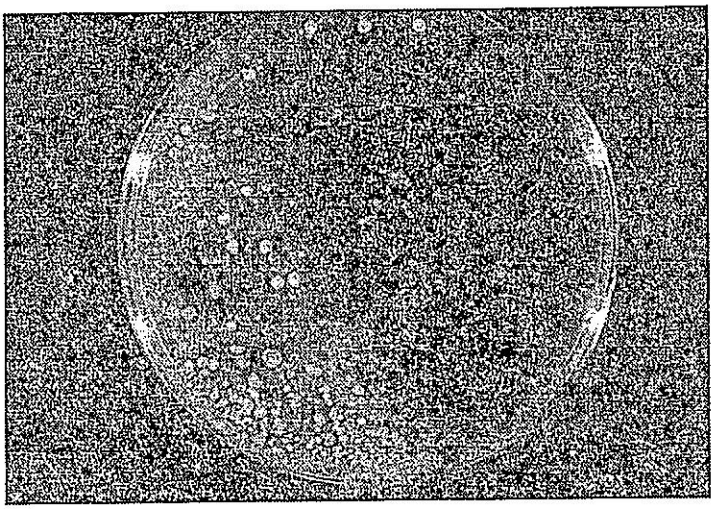

Fig. 3. P.multocida - 48$^{h}$ culture on nutrient agar 2

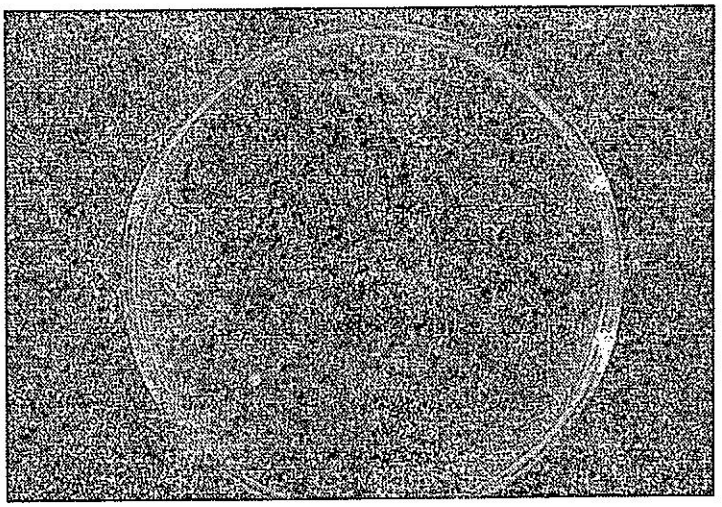

Fig. 4. P.multocida - 48$^{h}$ culture on nutrient agar 1

The highest percentage of positive findings (18,42%) was obtained on Columbia blood agar 2 when it contained bacitracin, gentamicin and clindamicyn. These combinations of chemotherapeutics inhibited the growth of accompanying bacterial flora and that facilitated the obtaining of pasteurellae in pure culture. Other selective media such as BA 2, NA 2, with the mentioned combinations of antimicrobial substances also expressed satisfactory degrees of selectivity.

Nutrient agar and blood agar, when they contained only bacitracin, gave the lowest percentage of isolation of P. multocida from nasal swabs of pigs. The prevalence of isolation for individual herds was variable. The percentage of positive findings varied from 7.14 to 34.28 % of the piglets regardless of the clinical atrophic rhinitis status of the farm. The strains of P. multocida isolated by culture methods were identified by the acriflavine test for determining capsular antigen type D. Out of 21 examined strains of P. multocida, 13 (i.e. 61.9 %) belonged to capsular type D.

Table 1. Isolation of P. multocida from nasal swabs from three herds applying six different selective media.

| Herd | No. exam. | Medium | | | | | | Max. No. of isolates | |
|---|---|---|---|---|---|---|---|---|---|
| | | BA-1 | BA-2 | NA-1 | NA-2 | CBA-1 | CBA-2 | | |
| | | P.m. | P.m. | P.m | P.m. | P.m. | P.m. | No. | % |
| I | 37 | 3 | 5 | 2 | 2 | 4 | 6 | 6 | 16,21 |
| II | 42 | 1 | 2 | 2 | 3 | 2 | 3 | 3 | 7,14 |
| III | 35 | 7 | ***10*** | 6 | 9 | 7 | ***12*** | 12 | 34,28 |
| | | 11 | 17 | 10 | 14 | 13 | 21 | 21 | 18,42 |
| Total | 114 | 9,65% | 14,91% | 8,77% | 12,28 | 11,40% | 18,42% | | |

Table 2. Comparison between culture media and the mouse inoculation method for isolating P. multocida from nasal cavities of piglets in herd III

| Medium | No. examined | Isolated strains | |
|---|---|---|---|
| | | No. | % |
| BA-2 | 35 | 10 | 28,57 |
| CBA-2 | 35 | 12 | 34,28 |
| Mouse inoc. | 35 | 6 | 17,14 |

Nasal swabs from 35 pigs from one herd (herd III with heavy clinical symptoms of atrophic rhinitis) were examined using BA 2, CBA 2 culture medium and the mouse inoculation method (Table 2.). P. multocida was isolated in 28.57% on BA 2, 34.28% on CBA 2 using selective medium, whereas the mouse

inoculation method yielded only 17.14% positive samples. Thus, the results obtained it could be concluded that Columbia blood agar 2 is the best method for isolating P. multocida.

## DISCUSSION

To make a diagnosis of atrophic rhinitis is a complex matter and it is made from a combination of data obtained by clinical and laboratory examinations. Although clinical signs of atrophic rhinitis are suggestive of infection, a definitive diagnosis is only possible after bacteriological examination of nasal secretions. B. bronchiseptica can be an important pathogen in young pigs, in which, acting alone, it is capable of inducing a moderately severe degree of turbinate discharge. The major importance of B. bronchiseptica infection in pig herds is the ability of this species to initiate turbinate damage and assist the colonisation of the nasal cavity by toxigenic strains of P. multocida. Attempts to isolate P. multocida from the nasal cavities of pigs directly on blood agar plates were not always successful. Toxigenic P. multocida grow readily in the laboratory but are difficult to isolate from nasal swabs because they are frequently overgrown by commensal flora. A perusal of the literature showed that various methods for isolation P. multocida have been described (Knight at al.,1983; Lariviere et al.,1993; Smith and Baskerville, 1983). Selective nutritive media containing antimicrobial agents have been developed to promote the isolation of P. multocida from nasal swabs (De Jong et al. 1985.)

Media incorporating polymyxin and gentamicin broadened the range of flora inhibited in comparison to those obtained from bacitracin alone. (Smith and Baskerville, 1983). Lariviere et al., (1992) reported that potassium tellurite in selective Knight medium was inhibitory to the selective growth of P. multocida from pigs, but the modified Knight medium without potassium tellurite proved to be the best simple method for isolating P. multocida. In our investigation the best results were obtained using Columbia blood agar which contained bacitracin, gentamicin and clindamycin.

Mouse inoculation seems to be the most commonly used method for the isolation of P. multocida Pedersen and Barford (1981) considered that bacteriological media are less sensitive than inoculation of the mouse for detecting P. multocida in material from the pig. However, the biological system is impractical on a large scale and therefore attempts were made to devise media simultaneously allowing easy selection and recognition of P. multocida. Hoffman et al., (1989.) compared Columbia blood agar supplemented with neomycin and bacitracin and the mouse inoculation method for effectiveness in recovering P. multocida from nasal swabs. They found that the mouse inoculation technique was most effective in the recovery of P. multocida. The results of our investigation indicate that the mouse inoculation test is not a definitive method for isolation of P. multocida. Lariviere et al., (1993.) also found that the mouse inoculation test was less sensitive than selective media for detecting P. multocida.

The prevalence of infection of toxigenic P. multocida is higher in herds with clinical disease (De Jong, 1992.). The organism can be present in 50-80% of weaned pigs in a herd with clinical disease in the finishing pigs. In their examinations Lariviere et al. (1993) isolated P. multocida in a high percentage (up to 100%), but did not find a marked difference in the prevalence P. multocida in

swine herds with or without clinical atrophic rhinitis. However, toxigenic isolates were found only in pigs with clinical atrophic rhinitis. In our investigation we found P. multocida in a considerably lower percentage than in these reports. Most of the isolates belonged to capsular type D. Bechmann and Schoss, (1988) examined tonsillar and nasal swabs from piglets from herds without clinical atrophic rhinitis for the presence of P. multocida. P. multocida was detected in 27.7% of the tonsillar swabs and in 18% of the nasal swabs. Cowart et al. (1989) reported that P. multocida was isolated more frequently from nasal swabs of 8-week-old pigs with higher atrophic rhinitis scores.

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## SELEKTIVNE PODLOGE ZA IZOLACIJU *Pasteurella multocida* IZ NOSNIH BRISEVA PRASADI.

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SADRŽAJ

Za izolaciju P.multocida iz nosnih briseva prasadi, koja potiču iz tri zapata sa kliničkim atrofičnim rinitisom, korišćene su selektivne hranljive podloge i test inokulacije na miševima.

U toku rada ispitano je 149 nosnih briseva. Svaki bris zasejan je na 6 različitih selektivnih podloga koje su sadržavale različite kombinacije hemoterapeutika: bacitracin, gentamicin, polimyxin B, clindamicin. Selektivnost hranljivih podloga bila je uslovljena sadržajem i koncentracijom hemoterapeutika. Najveći procenat pozitivnih nalaza (18,42%) dobijen je na Kolumbija krvnom agaru 2, koji je sadržavao bacitracin, gentamicin i klindamicin. Stepen izolacije P.multocida varirao je u okviru različitih zapata i kretao se od 7.14 do 34.28 %. Primenom akriflavin testa ustanovili smo da 61,90% izolovanih sojeva P. multocida pripada tipu D. Testom inokulacije na miševima, P.multocida je izolovana u znatno nižem procentu (17,14%), u odnosu na selektivne podloge, krvni agar 2 (28,57%) i Kolumbija krvni agar 2 (34,28%).