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SELECTIVE MEDIA FOR THE ISOLATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* FROM THE PIG

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Selective media were developed and evaluated for isolation of A. pleuropneumoniae from pig tonsils. Samples were obtained from four pig herds with a clinical history of pleuropneumonia. For isolation of A. pleuropneumoniae 93 pig tonsils were collected at slaughter. Each sample was streaked on to four different selective media (modified PPLO agar (mPPLO), Brain-Heart agar (BH), Columbia agar (CA), Miller-Hinton chocolate agar (MHCA)) containing different combinations of antibiotics, NAD and nystatin. The selectivity of nutritive media is conditioned by the content of antibiotics, as well as by the type of medium used. Mean isolation rate of A. pleuropneumoniae in the investigated herds, was 17.2%. The best results were obtained using $PPLO_2$ agar, 20.4%. The other media supplemented with the mentioned antibiotics gave satisfactory results.

Key words: A. pleuropneumoniae, selective media, pig

INTRODUCTION

Pleuropneumonia is one of the important bacterial diseases of the porcine respiratory tract. Pneumonia often develops a chronic course of clinical illness or occurs as a subclinical disease, but may result in death of the animal, causing serious losses such as reduced production or increased medication or vaccination costs. Pleuropneumonia of pigs is widespread in countries with intensive pig production, causing heavy losses to the pig industry.

Actinobacillus pleuropneumoniae is the etiologic agent of fibrinous, hemorrhagic and necrotizing pleuropneumonia of pigs (Nielsen, 1974). It is the etiologic agent in bronchial or nasal exudate and pneumonic lesions from freshly dead animals. *A. pleuropneumoniae* is a small, Gram-negative, encapsulated rod with typical coccobacillary morphology. The organism does not grow on blood agar unless it is supplemented with NAD and shows satellitism around colonies of staphylococci. Staphylococcal streaks are normally required for primary isolation on this medium. The organism forms colonies 0.5-1 mm after incubation for 24 hours on blood agar in the presence of staphylococcal colonies and is beta-hemolytic, particularly when sheep red blood cells are used. *A. pleuropneumoniae* produces an increased zone of hemolysis within the zone of

partial lysis surrounding a beta-toxinogenic *Staphylococcus aureus* (the CAMP phenomenon) (Nicolet, 1970; Kilian, 1976). This CAMP phenomenon has been shown to be related to the possession of the three cytolysins ApxI, ApxII, and ApxIII (Frey *et al*., 1994; Jansen *et al*,. 1995). Gram-stained smears of lung lesions contain numerous gram-negative coccobacilli.Their identity can be confirmed as *A. pleuropneumoniae* by using a fluorescent antibody test (Nicolet, 1970), by immunoperoxidase, by detection of serovar-specific antigens in lung extracts with a coagglulination test, by using a latex agglutination test, or by enzyme-linked immunosorbent assay (ELISA). Nucleic acid from bacteria may be detected by a number of methods, including labeled DNA probes in tissue and the polymerase chain reaction (PCR). Direct confirmation by PCR of the presence of *A. pleuropneumoniae* in tissue is not yet routine.

Surveillance programs in pig herds usually rely on serological methods, e.g. the complement fixation test and ELISA. However, serological tests are currently serotype specific, i.e. limited to the detection of one or a few of the 12 serotypes described so far. It has been demonstrated that serologically negative pigs can harbor *A. pleuropneumoniae* in the oropharyngeal tonsils (Möller *et al*., 1993; Sidebe *et al*., 1993), and *A. pleuropneumoniae* may be isolated from the tonsils in a fairly high percentage of pigs at slaughter (Möller *et al*., 1993). A number of authors have emphasized the problem of isolation of *A. pleuropneumoniae*. Therefore, cultivation is an important adjunct to serology for surveillance and diagnosis of *A. pleuropneumoniae* infections. Since A. *pleuropneumoniae* is a fastidious organism, and is easily overgrown by other bacteria from the nasopharyngeal cavity (Gilbride and Rosendal, 1983) selective media for isolation from tonsils are of great value. Investigations were mostly aimed towards finding selective media by the addition of antibiotics and chemotherapeutics that would inhibit the growth of accompanying flora without harming the growth of *A. pleuropneumoniae* at the same time.

A. pleuropneumoniae is particularly susceptible *in vitro* to penicillin, ampicillin, cephalosporin, chloramphenicol, tetracyclines, colistin, sulfonamide, cotrimoxazole (trimethoprim + sulfamethoxazole), and gentamicin, to which it has low minimum inhibitory concentrations (MIC). High MIC values were found for streptomycin, kanamycin, spectinomycin, spiramycin, and lincomycin (Nadeau *et al*,. 1988). *A. pleuropneumoniae* has been found to be highly resistant to bacitracin (Shimizu *et al*., 1982) and lincomycin (Nadeau *et al*., 1988; Eaves *et al*., 1989) and these antibiotics alone or together with crystal violet have been used in previously described selective media (Csukas, 1980; Gilbride and Rosendal, 1983; Sidebe *et al*., 1993).

The aim of the present study was to develop an improved selective medium for *A. pleuropneumoniae*.

MATERIALS AND METHODS

Pigs: A total of 93 pigs originating from four farms with evidence of pleuropneumonia were used in this study.

Samples: Tonsils. A total of 93 pig tonsils were randomly selected at the slaughterhouse. The tonsils were stored at 5°C overnight, then seared with a hot iron on the surface and cut open to obtain scrapings of approximately 100 μl from the tonsil tissue. Each scraping was suspended in 0.6 ml phosphate buffered saline (PBS) and mixed. Fifty μl of this suspension was spread with a triangle spatula on each of the four media under test: modified PPLO agar (mPPLO), Brain-heart agar (BH), Columbia agar (CA), Miller-Hinton chocolate agar (MHCA), each with and without supplements of inhibitory substance. The plates were incubated at 37°C for 24 - 72 hours with prolonged incubation up to 96 hours.

Bacterial strains: In this study eight strains of *A. pleuropneumoniae* isolated from pigs with a history of pleuropneumonia, as well as three reference strains: CAPM Brno (5870, 3800 and 3648) were used. The strains were lyophilized. Before use the isolates were passaged twice on PPLO agar, each time at 37°C in a normal atmosphere with NAD. The sensitivity of the isolated field strains and reference strains was examined prior to the addition of therapeutics.

A suspension in saline of each strain of *A. pleuropneumoniae* was standardized to a McFarland value of 0.5 (1.5 X 10^8 CPU/ ml). The suspensions were simultaneously inoculated on the media with and without antimicrobial additives. After incubation at 37°C for 48-72 h the plates were visually evaluated for growth. Based upon these results we used the following antibiotics: bacitracin and lincomycin.

Media: The following media were investigated:

• Modified PPLO agar:	PPLO agar (Difco)	34.0 g
	Glucose	1.0 g
	Distilled water	900 ml

After sterilization, the agar was allowed to cool to 45-50 °C and inactivated horse serum (50.0 ml) and fresh yeast extract (100.0 ml) were added.

• Brain-heart agar:	B-H infusion	37.0 g
	Tryptone	10.0 g
	Agar	18.0 g
	Distilled water	1000 ml
• Columbia agar:	Commercially available (Biolife)	
• Miller-Hinton chocolate agar:	Commercially available (Biolife)	

The agar was based on Miller-Hinton agar (MH) with 10% heated horse blood (80°C for 10 min).

The nutritive media contained antibiotics and NAD in the following concentrations:

Modified PPLO agar	1. no inhibitory compounds 2. Bacitracin (4 IU/ml) + Lincomycin (1 μg/ml) + Nystatin (50 μg/ml)
Brain-heart agar	1. no inhibitory compounds 2. Bacitracin (2 IU/ml) + Lincomycin (1 μg/ml) + Nystatin (50 μg/ml)
Columbia agar	1. no inhibitory compounds 2. Bacitracin (3 IU/ml) + Lincomycin (2 μg/ml) + Nystatin (50 μg/ml)
Miller-Hinton chocolate agar	1. no inhibitory compounds 2. Bacitracin (3 IU/ml) + Lincomycin (2 μg/ml) + Nystatin (50 μg/ml)

All media were supplemented with 0.0025 % NAD (Sigma). Substances were added when the molten agar was cooled for about 50 ^{o}C. Solutions of antimicrobial agents were freshly prepared and sterilized by filtration. Media prepared in such a way were kept at 4 ^{o}C and were used within 7 days after preparation.

Characterization of isolates: Colonies were selected according to their morphological characteristics on different media and subjected to a series of different biochemical tests: oxidase, mannitol, glucose, urease, beta-galactosidase (ONPG), catalase, indole, arabinose, raffinose, sorbitol and inositol.

From each inoculated plate smooth, gray and transparent colonies were transferred to PPLO blood agar to determine their hemolytic activity. Isolates showing haemolysis were confirmed morphologically by Gram staining and the CAMP reaction. Biochemically, all isolates that were positive in the following tests: oxidase, mannitol, glucose, urease and beta-galactosidase (ONPG), and negative in the following tests: catalase, indole, arabinose, raffinose, sorbitol and inositol were accepted as *A. pleuropneumoniae*.

RESULTS AND DISCUSSION

The results of the examinations of 93 pig tonsils streacked on four different media containing different combinations of antibiotics are presented in Table 1. In our preliminary examination we noticed that an increase in the amount of bacitracin from 2 to 4 IU did not affect the growth of A. pleuropneumoniae or the appearance of colonies, nor did it significantly inhibit other bacterial flora.

Between 12 and 41 tonsils were tested from each of the four herds. *A. pleuropneumoniae* was found in all four herds and the mean isolation rate was 17.2%. *A. pleuropneumoniae* could be isolated in all the investigated media.The highest percentage of positive findings was obtained on $PPLO_2$ and on $MHCA_2$ (20.4 – 15.1%). These media also showed the highest degree of isolation of *A. pleuropneumonia* in pure culture. Neither the selective media nor the non-selective media prevented Proteus species from growing.

Table 1. The effect of different media on the isolation rate of *A.pleuropneumoniae*

Medium		*A.pp*	*A.pp* other	Other bacteria	Ø	Isolation	
						No.	%
Modified PPLO agar	1.	–	6	87	–	6	6.5
	2.	3	16	71	3	19	20.4
Brain-heart agar	1.	–	4	89	–	4	4.3
	2.	1	11	76	5	12	12.9
Columbia agar	1.	–	3	9o	–	3	3.2
	2.	–	7	86	–	7	7.5
Miller-Hinton chocolate agar	1.	–	5	88	–	5	5.4
	2.	2	12	73	6	14	15.1

Bacteriological diagnosis of *A. pleuropneumoniae* infections has traditionally been based on its isolation from lungs (Nicolet *et al*., 1971). Isolation of *A. pleuropneumoniae* from acute lung lesions does not require a selective medium since *A. pleuropneumoniae* in such cultures are relatively abundant and free from contaminating flora. However, in an effective surveillance programme it is important to diagnose subclinical infections with *A. pleuropneumoniae* as seroconversion may be the first sign of infection even though clinical signs are absent. In this case detection of the organism in tonsils is also necessary for confirmation of the serological diagnosis. Although isolation and culture of *A. pleuropneumoniae* is necessary for a definitive diagnosis, other tests based on immunological or DNA methods may be more rapid and cheaper for surveillance programs.

Different media have been used for detecting *A. pleuropneumoniae* from pig tonsils. Gilbride and Rosendal (1983) isolated *A. pleuropneumoniae* from 5% of the samples when culturing the cut surface of tonsils of 80 slaughtered pigs on TSA agar plates supplemented with lincomycin, crystal violet, bacitracin and NAD. Sidibe *et al*. (1993) found that PPLO-agar supplemented with 1 μg/ml lincomycin, 2 μg/ml crystal violet, 156 μg/ml bacitracin and 0.0001% NAD was more effective for isolation of *A. pleuropneumoniae* from tonsils than blood-agar based on TS A and supplemented with lincomycin, crystal violet, bacitracin and NAD. The relative number of pigs positive for isolation of *A. pleuropneumoniae* was 24% when culturing samles from 429 pigs from 15 herds on both media. Moler *et al*. (1993) showed that a chocolate agar similar to the BAB medium used in the present study, but supplemented with 300 μg/ml bacitracin, was better for isolation of *A. pleuropneumoniae* than blood agar containing 300 μg/ml bacitracin. When culturing tonsils from 303 slaughtered pigs on chocolate agar with 300 μg/ml bacitracin, they found that 22% of the pigs were carrying *A. pleuropneumoniae*.

In the present study the selective and NAD supplemented medium $PPLO_2$ was the most sensitive medium for the isolation of *A. pleuropneumoniae* from pig tonsils. On this medium *A. pleuropneumoniae* was isolated from 20.4% of the pigs, whereas only 6.5% of the pigs were *A. pleuropneumoniae* culture positive when the medium $PPLO_1$ without antibiotics but with NAD was used. The prevalence of *A. pleuropneumoniae* carrying pigs in the present study was comparable to those observed in other studies (Moler *et al*., 1993; Sidebe *et al*., 1993). However, the sample size is too small to give a precise estimate of the overall prevalence of *A. pleuropneumoniae* carrying pigs.

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SELEKTIVNE PODLOGE ZA IZOLACIJU *ACTINOBACILLUS PLEUROPNEUMONIAE* KOD SVINJA

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SADRŽAJ

Za izolaciju *A. pleuropneumoniae* iz tonzila svinja pripremljene su i ispitane različite selektivne podloge. Uzorci su uzeti od svinja sa 4 farme, gde je utvrđena pleuropneumonija. Za izolaciju *A. pleuropneumoniae* iz 93 tonzila svinja uzeti su uzorci na liniji klanja. Svaki uzorak je bio zasejan na 4 različite hranljive podloge (modifikovani PPLO agar, moždano-srčani agar, Kolumbija agar i Miller-Hinton čokoladni agar), koje su sadržavale različite kombinacije antibiotika, NAD faktor i nistatin. Selektvnost hranljivih podloga bila je uslovljena sadržajem antibiotika i vrstom korišćene podloge. Prosečni stepen izolacije *A. pleuropneumoniae* u ispitivanim zapatima iznosio je 17.2%. Najbolji rezultati su postignuti upotrebom $PPLO_2$ agara, (20.4%). Ostale podloge sa pomenutim kombinacijama antibiotika pokazale su zadovoljavajuće rezultate.