AN ATTEMPT TO IMPROVE BOVINE *MYCOPLASMATALES'* PRIMARY ISOLATION[1]

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ABSTRACT - The sensitivity to 30 antibiotics of *Mycoplasma* (10 strains) and *Acholeplasma* (8 strains) was tested in an attempt to find a better one for suppression of contamination in the culture media, used for primary isolation of *Mycoplasmatales*. The most effective antibiotics for suppression of Gram-positive and Gram-negative organisms and did not inhibit *Mycoplasmatales* were: carbenicillin, vancomycin and fosfomycin. Although none of the antibacterial drugs that are inhibitors of cell wall synthesis was active against *Mycoplasmatales*, the majority of them were also ineffective against Gram-positive and/or Gram-negative contaminant bacteria.

Index terms: *Mycoplasma*, *Acholeplasma*, antibiotics, Gram-positive, Gram-negative, carbenicillin, vancomycin, fosfomycin, bacteria

UMA TENTATIVA PARA MELHORIA DO ISOLAMENTO INICIAL DE *MYCOPLASMATALES* DE BOVINOS

RESUMO - A sensibilidade a 30 antibióticos apresentada por *Mycoplasma* (10 amostras) e *Acholeplasma* (8 amostras) foi avaliada, numa tentativa de se encontrar um antibiótico mais eficaz para suprimir a contaminação secundária, no meio de cultura utilizado para o isolamento primário de *Mycoplasmatales*. Os antibióticos que se mostraram mais eficazes para supressão do crescimento de organismos Gram-positivos e Gram-negativos, e que não inibiram *Mycoplasmatales* foram: carbenicilina, vancomicina e fosfomicina. Apesar de nenhuma das drogas antibacterianas, que agem como inibidores da síntese da parede celular, ter sido ativa contra *Mycoplasmatales*, a maioria delas foi também ineficaz contra as bactérias Gram-positivas e/ou Gram-negativas contaminantes.

Termos para indexação: *Mycoplasma*, *Acholeplasma*, antibióticos, Gram-positivo, Gram-negativo, carbenicilina, vancomicina, fosfomicina, bactéria.

INTRODUCTION

One of the most important characteristics of the organisms belonging to the Class *Mollicutes* is their lack of a defined cell wall, which makes them incapable of peptidoglycan's synthesis and its precursors, and consequently resistant to penicillin and analogues that inhibit cell wall synthesis (Stanier et al., 1987). The resistance of *Mycoplasmatales* to penicillin made it possible the introduction of this antibiotic in the culture medium employed for primary isolation and cultivation of these organisms, and proved to be very useful for suppression of bacterial contamination (Hayflick, 1965).

At present, with increasing number of mycoplasma species isolated from different anatomical sites, it seems that the procedures for mycoplasma's isolation should be reviewed in an attempt to recover a larger number of these organisms, when contaminated materials have to be cultured (International Organization for Mycoplasmology, 1992).

The sensitivity *in vitro* of *Mycoplasma* and *Acholeplasma* strains, isolated from bovine sam-

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ples and tested against antibacterial drugs, was described by Liberal & Boughton (1991). The results obtained could be useful either for clinical applications or for *in vitro* research about isolation procedures.

On the basis of these findings, the present research was carried out with strains of *Mycoplasmatales* and contaminant bacteria isolated from nasal discharges of calves (without clinical signs of respiratory infection), to find a better antibiotic (or combination of) than ampicillin to be introduced in the culture medium E broth (a modified Eaton broth) (Roberts & Pijoan, 1971), used for bovine mycoplasma primary isolation.

MATERIALS AND METHODS

A total of 144 samples (nasal swabs) was collected from the upper respiratory tract of apparently healthy calves. The nasal samples were collected using commercial dry sterile flexible cotton swabs, which were cut off into a tube containing 3 ml of E broth (Roberts & Pijoan, 1971). The tubes were incubated aerobically at 37°C and checked for change in pH and/or turbidity after 24 hours. If either was observed, one drop of the broth was subcultured on to a fresh E broth; another drop was subcultured to an ordinary blood agar and to Teague agar using the methodology described by Liberal (1988); and the original broth was filtered through a 0.45 μm disposable filter, to a fresh E broth tube. All subcultures were kept aerobically at 37°C and checked for growth after each 24 hours, up to one week (Razin & Tully, 1983).

Mycoplasmatales

When growth was observed on E broth (turbidity or change in pH) one drop of the broth was subcultured on an E plate (E broth added with 1% of purified agar, without phenol red), using the running drop technique. Plates were kept at 37°C in an 5% CO_2 atmosphere incubator, observed each 24 hours for mycoplasma - type colonies ("fried-egg") with a plate microscope. When typical "fried-egg" colonies were observed, a block of agar containing some colonies were cut from the plate, and placed in a fresh E broth tube (Razin & Tully, 1983). The "cloning" procedure was repeated three times, when the mycoplasmas isolated were finally identified by biochemical (dependence on sterols and glucose fermentation) (Razin & Tully 1983), and serological tests (growth inhibition test and film inhibition test) (Clyde Junior, 1964; Thorns & Boughton 1978).

Mycoplasmatales

The number of strains to be tested was selected arbitrarily, and 4 out of 7 *M. bovis* strains and 4 out of 13 *A. laidlawii* strains, isolated from the field (nasal discharges), were chosen at random to perform the test. Two *M. bovis* strains (OB 823; 8B 70) and two *A. laidlawii* strains (8M 2713; 8M 1661) from The Central Veterinary Laboratory Mycoplasma Section Culture Collection (The Central Veterinary Laboratory, New Haw, Weybridge, England) were selected to be tested. *M. bovirhinis* (9M 1019; 7B 10); *M. dispar* (NCTC 10125); *M. bovoculi* (M 165/69); *A. oculi* (NCTC 10150); and *A. axanthum* (3B 256) were also introduced in the study, as they are organisms likely to be isolated fron nasal discharges of calves.

Contaminant bacteria

When growth was observed on blood agar and/or Teague agar plates, a smear was prepared and Gram staining was used for a first evaluation of the organisms present in the sample. From strains grown on blood agar the presence of haemolysis, colony pigmentation, size and type were observed. For colonies grown in Teague agar, the presence of lactose fermenting organisms, and colony size and type were also observed. Biochemical tests were used for identification of the organisms following the recommendation of the Bergey's Manual of Systematic Bacteriology (Krieg & Bolt, 1984; Sneath et al., 1986).

For the most frequent bacteria isolated, a range between 18 and 30% of strains of each bacterium was chosen at random, i.e., *Escherichia coli* = 17 strains; *Klebsiella pneumoniae* = 7 strains; *Bacillus subtilis* = 6 strains ; *Streptococcus* sp. = 5 strains; and *Serratia marcescens* = 2 strains. For the remaining bacteria between 50 and 100 per cent of the strains isolated in this study were used.

Antibiotic sensitivity tests

The tests were carried out using commercially available impregnated discs of the following antibacterial drugs: amikacin (30 μg), ampicillin (10 μg), carbenicillin (100 μg), cephalexin (30 μg), cephalothin (30 μg), cephazolin (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), fosfomycin (50 μg), gentamycin (10 μg), kanamycin (30 μg),

lincomycin (15 µg), nalidixic acid (30 µg), netilmycin (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), oxacillin (5 µg), penicillin (10 µg), pipemidic acid (20 µg), rifampicin (30 µg), sisomycin (10 µg), sulphazotrim (25 µg), sulphonamide (300 µg), tetracycline (30 µg), tobramycin (10 µg) and vancomycin (30 µg). The technique used to perform the sensitivity test was the one preconizad by Bauer et al. (1966) using Mueller - Hinton agar medium for contaminant bacteria. For *Mycoplasmatales*, a modified E agar (without ampicillin and phenol red) was used, following Liberal & Boughton (1991). With the results observed in that experiment, a range of antibiotics that showed no action against *Mycoplasmatales* was selected, and the minimum inhibitory concentration test (MIC) (Washington & Barry, 1974) was performed with the contaminants, in an attempt to find a better antibiotic to suppress the bacterial contamination. As the susceptibility of a given bacterial species may be influenced by the pH, the incubation atmosphere, and by the protein concentration, the MIC tests were performed using the same medium (E broth and E plates from which ampicillin was omitted), the same incubation (37°C in a 5% CO_2 atmosphere), and the same inoculum size (0,5 ml aliquots diluted at 1/100).

The interpretation of the results followed the same arbitrary criteria described previously by Liberal & Boughton (1991) where: Sensitive (S) means an inhibition zone equal or higher than 10 mm; Intermediate (I) means an inhibition zone smaller than 10 mm; and Resistant (R) means that no inhibition zone at all was observed.

RESULTS AND DISCUSSION

As the main objective of this study was to find a better antibiotic for inclusion in E broth, to enhance *Mycoplasmatales* isolation from calves, any inhibition produced by the antibiotic eliminated it from consideration. Strains other than those isolated from these nasal discharges were tested as well, covering a wider range of *Mycoplasmatales*, to ensure the success of further isolations from field and laboratory strains.

In this research, *M. bovis* (7 strains) and *A. laidlawii* (13 strains) were isolated from 13.2 percent of the calves sampled. Representatives of *Enterobacteriaceae* were the most frequent bacteria isolated from the nasal discharges: *Escherichia coli* (63.2%) and *Klebsiella pneumoniae* (27.1%), followed by representatives of the Gram-positive group: *Bacillus subtilis* (20.1%) and *Streptococcus* sp. (10.4%). The other bacteria isolated were *Serratia marcescens* (7.6%), *Acinetobacter calcoaceticus* (5.6%), *Hafnia alvei* (3.5%), *Staphylococcus epidermidis* (2.1%), *Enterobacter cloacae* (1.4%), *Sarcina* sp. (1.4%), *Staphylococcus aureus* (0.7%), and *Pseudomonas aeruginosa* (0.7%).

The results of the sensitivity "in vitro" obtained for *Mycoplasma* spp.; *Acholeplasma* spp.; and contaminant bacteria are shown in Tables 1, 2 and 3 respectively.

In an attempt to choose antibacterial drugs active against both Gram-negative and Gram--positive bacteria but not against *Mycoplas-*

TABLE 1. Antibacterial drugs tested *in vitro* against *mycoplasma* species.

Antibiotic	Sensitivity results			Total	I+S	Percentage I+S %
	Resistant	Intermediate	Sensitive			
Amikacin	2	7	1	10	8	10.0	80.0
Ampicillin	10	0	0	10	0	0	0
Carbenicillin	10	0	0	10	0	0	0
Cephalexin	10	0	0	10	0	0	0
Cephalothin	10	0	0	10	0	0	0
Cephazolin	10	0	0	10	0	0	0
Cefotaxime	10	0	0	10	0	0	0
Cefoxitin	10	0	0	10	0	0	0
Ceftriaxone	10	0	0	10	0	0	0
Clindamycin	0	0	10	10	10	100.0	100.0
Chloramphenicol	0	0	10	10	10	100.0	100.0
Erythromycin	9	1	0	10	1	0	10.0
Fosfomycin	10	0	0	10	0	0	0
Gentamycin	0	7	3	10	10	30.0	100.0
Kanamycin	0	8	2	10	10	20.0	100.0
Lincomycin	0	3	7	10	10	70.0	100.0
Nalidixic Acid	7	2	1	10	3	10.0	30.0
Netilmycin	0	7	3	10	10	30.0	100.0
Nitrofurantoin	0	0	10	10	10	100.0	100.0
Norfloxacin	0	5	5	10	10	50.0	100.0
Oxacillin	10	0	0	10	0	0	0
Penicillin	10	0	0	10	0	0	0
Pipemidic Acid	9	1	0	10	1	0	10.0
Rifampicin	10	0	0	10	0	0	0
Sisomycin	9	1	0	10	1	0	10.0
Sulphazotrim	9	1	0	10	1	0	10.0
Sulphonamide	10	0	0	10	0	0	0
Tetracycline	0	0	10	10	0	100.0	100.0
Tobramycin	10	0	0	10	0	0	0
Vancomycin	10	0	0	10	0	0	0
Total of tests	205	43	52	300	95	17.3	31.7

I = Intermediate
S = Sensitive
Mycoplasma species tested = *M. bovis*; *M. bovirhinis*; *M. bovoculi*; and *M. dispar*.

TABLE 2. Antibacterial drugs tested *in vitro* against *acholeplasma* species.

Antibiotic	Sensitivity results			Total	I+S	Percentage sensitive	I+S %
	Resistant	Intermediate	Sensitive				
Amikacin	0	4	4	8	8	50.0	100.0
Ampicillin	8	0	0	8	0	0	0
Carbenicillin	8	0	0	8	0	0	0
Cephalexin	8	0	0	8	0	0	0
Cephalothin	8	0	0	8	0	0	0
Cephazolin	8	0	0	8	0	0	0
Cefotaxime	8	0	0	8	0	0	0
Cefoxitin	8	0	0	8	0	0	0
Ceftriaxone	8	0	0	8	0	0	0
Clindamycin	0	0	8	8	8	100.0	100.0
Chloramphenicol	0	0	8	8	8	100.0	100.0
Erythromycin	0	0	8	8	8	100.0	100.0
Fosfomycin	8	0	0	8	0	0	0
Gentamycin	0	6	2	8	8	25.0	100.0
Kanamycin	1	6	1	8	7	12.5	87.5
Lincomycin	0	0	8	8	8	100.0	100.0
Nalidixic Acid	7	1	0	8	1	0	12.5
Netilmycin	0	5	3	8	8	37.5	100.0
Nitrofurantoin	0	0	8	8	8	100.0	100.0
Norfloxacin	0	2	6	8	8	75.0	100.0
Oxacillin	8	0	0	8	0	0	0
Penicillin	8	0	0	8	0	0	0
Pipemidic Acid	7	1	0	8	1	0	12.5
Rifampicin	0	0	8	8	8	100.0	100.0
Sisomycin	8	0	0	8	0	0	0
Sulphazotrim	6	1	1	8	2	12.5	25.0
Sulphonamide	8	0	0	8	0	0	0
Tetracycline	0	0	8	8	8	100.0	100.0
Tobramycin	8	0	0	8	0	0	0
Vancomycin	8	0	0	8	0	0	0
Total of tests	141	26	73	240	99	30.4	41.3

I = Intermediate
S = Sensitive
Acholeplasma species tested = *A. laidlawii*; *A. axanthum*; and *A. oculi*.

TABLE 3. Sensitivity *in vitro* of Gram-negative and Gram-positive bacteria to 30 different antibacterial drugs.

Antibiotic	Sensitivity results			Total of samples tested
	Resistant	Intermediate	Sensitive	
Amikacin	29	3	19	51
Ampicillin	45	-	6	51
Carbenicillin	43	3	5	51
Cephalexin	18	1	9	28
Cephalothin	33	4	14	51
Cephazolin	20	1	2	23
Cefotaxime	23	4	24	51
Cefoxitin	43	5	3	51
Ceftriaxone	9	-	19	28
Clindamycin	21	-	2	23
Chloramphenicol	15	2	34	51
Erythromycin	38	-	13	51
Fosfomycin	10	6	7	23
Gentamycin	16	2	33	51
Kanamycin	12	6	5	23
Lincomycin	28	-	-	28
Nalidixic Acid	29	4	18	51
Netilmycin	17	-	34	51
Nitrofurantoin	36	2	13	51
Norfloxacin	3	-	25	28
Oxacillin	46	-	5	51
Penicillin	50	-	1	51
Pipemidic Acid	18	2	3	23
Rifampicin	29	-	4	23
Sisomycin	5	-	23	28
Sulphazotrim	31	-	20	51
Sulphonamide	13	-	5	18
Tetracycline	30	9	12	51
Tobramycin	22	1	28	51
Vancomycin	39	-	12	51
Total of sensitivity tests done	771	55	398	1224

Gram-Negative bacteria tested = *Escherichia coli*; *Klebsiella pneumoniae*; *Serratia marcescens*; *Acinetobacter calcoaceticus*; *Hafnia alvei*; *Enterobacter cloacae*; and *Pseudonas aeruginosa*.
Gram-Positive bacteria tested = *Bacillus subtilis*; *Streptococcus* sp.; *Staphylococcus epidermidis*; and *Staphylococcus aureus*.

matales, an arbitrary level of sensitivity of 35% or higher (Liberal & Boughton, 1991) was selected as a limit. MIC tests (Washington & Barry, 1974) were carried out for the contaminant bacteria isolated, using the following antibiotics (selected as previously described): tobramycin, carbenicillin, cephalothin and fosfomycin. The final results are shown in Table 4.

As expected none of the antibacterial drugs that act as inhibitors of cell wall synthesis was active against *Mycoplasmatales*. The group was represented by the penicillins (penicillin G, oxacillin, ampicillin and carbenicillin); the cephalosporins (cephalothin, cephazolin, cephalexin, cefotaxime and ceftriaxone); the cephamycin (cefoxitin); and the antibacterial antibiotics vancomycin and fosfomycin. An inhibitor of protein synthesis, tobramycin, was also not active against *Mycoplasmatales*. Sisomycin (another inhibitor of protein synthesis) and rifampicin (an inhibitor of nucleic acid synthesis) showed variable results for *Myco-*

TABLE 4. Minimum inhibitory concentration of antibacterial drugs from Gram-negative and Gram-positive bacteria.

Antibiotic	Gram-negative bacteria	Gram-positive bacteria	Observation
Carbenicillin	15.000 µg/ml	15.000 µg/ml	*P. aeruginosa* and *B. subtilis* were resistant
Fosfomycin	60.000 µg/ml	3.750 µg/ml	*B. subtilis* was resistant
Tobramycin	46.500 µg/ml	46.500 µg/ml	pH very acid
Vancomycin	31.200 µg/ml	7.800 µg/ml	*P. aeruginosa* and *B. subtilis* were resistant

plasma and *Acholeplasma* species, which preclude them to be included in the medium.

The most effective antibiotic against Gram--negative bacteria was gentamycin, and for Gram--positive bacteria was erythromycin. Unfortunately, both drugs were also effective against *Mycoplasmatales:* gentamycin being active for 100% of the strains tested (Intermediate + Sensitive result), and erythromycin showed 10% and 100% (Intermediate + Sensitive results) for *Mycoplasma* and *Acholeplasma* species respectively. Chloramphenicol showed activity for 69,4% and 60% of the Gram-negative and Gram-positive strains tested, but it was also active against *Mycoplasmatales* (Intermediate + Sensitive = 100%).

For contaminant bacteria the results were expressed as MIC, which was the lowest concentration of drug inhibiting colour change in broth by the test organism, and from which viable organisms were recovered after subculture onto E plates. For *Mycoplasmatales* the results were expressed as the highest concentration of antibiotic allowing the growth of the organisms, when subcultured on E plates.

Tobramycin was not indicated for incorporation in the medium because it tends to make the pH very acid even in a concentration of 46.500 ug/ml, when a poor growth of *Mycoplasmatales* was obtained. With cephalothin the results showed bacterial resistance in concentrations up to 56.700 µg/ml and no further test was carried out.

CONCLUSIONS

With the results obtained it is believed that, the best alternative antibiotics to be used instead of or as well as ampicillin in E broth is carbenicillin (15.000 µg/ml), vancomycin (31.200 µg/ml) or fosfomycin (60.000 µg/ml), as they were inactive against all *Mycoplasmatales* strains under study, and they were able to suppress the growth of the majority of Gram-negative and Gram-positive bacteria tested.

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