ANTIMICROBIAL RESISTANCE IN BOVINE COMMENSAL NASAL PASTEURELLACEAE

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**ABSTRACT**

The presence of Pasteurellaceae in the nasopharynx of 57 (extensively housed) clinically healthy calves was investigated with special reference to the recent reclassification within this bacterial family and tetracycline resistance. A total of 40 strains belonging to the family Pasteurellaceae were isolated in 34 calves (59.6%) out of 10 herds (76.9%). Identification of the strains resulted mainly in Pasteurella (P.) multocida (n=35). Other bacteria were P. canis (1), P. trehalosi (1), Mannheimia (M.) varigena (2) and one untypable Mannheimia spp. Mannheimia haemolytica senso stricto was not isolated. Overall antimicrobial resistance was rare. In five tetracycline resistant P. multocida strains originating from the same farm no plasmid DNA was detected, suggesting a chromosomal localisation and a clonal spread of the underlying resistance determinants. In summary, antimicrobial susceptible P. multocida strains were the predominant Pasteurellaceae present in the nasopharynx of young extensively housed calves.

**SAMENVATTING**

In 57 gezonde en extensief gehuisveste kalveren werd het voorkomen van Pasteurellaceae en hun respectievelijke antibioticumresistentiepatronen onderzocht. Hierbij werd speciale aandacht besteed aan tetracyclineresistentie en aan de recente omvorming van [Pasteurella] haemolytica naar Mannheimia haemolytica. In totaal werden 40 Pasteurellaceae geïsoleerd in 34 kalveren (59,6%) op 10 bedrijven (76,9%). Pasteurella multocida werd het meest geïsoleerd (n=35), naast andere Pasteurella (n=2) en Mannheimia species (n=3). Mannheimia haemolytica senso stricto werd niet gevonden. Tetracyclineresistentie werd gedetecteerd in 5 Pasteurella multocida stammen afkomstig van hetzelfde bedrijf, maar kon niet worden geassocieerd met de aanwezigheid van plasmidair (horizontaal overdraagbaar) genetisch materiaal. Samenvattend werden voornamelijk antibioticumgevoelige P. multocida stammen geïsoleerd uit de neusflora van de extensief gehuisveste kalveren.

1. INTRODUCTION

Bovine respiratory disease (BRD) has been attributed to be the syndrome having the highest morbidity and mortality in calves [7]. [Pasteurella] haemolytica and P. multocida, both belonging to the family Pasteurellaceae, are known to act as primary or secondary (opportunistic) pathogen in the BRD-complex. Older studies have shown that both are present in the upper respiratory tract of healthy and infected calves [1]. In 1999, the reclassification of the trehalose negative [Pasteurella] haemolytica complex by means of an extensive polyphasic study has led to the new genus Mannheimia. Herein, Mannheimia (M.) haemolytica has been defined the type species, besides four so far designated other species [2]. The commensal behaviour of these organisms also implies that they can harbour resistance determinants which may be transferred horizontally into pathogenic bacteria and consequently cause therapy failure [4, 10].

The aim of the present study was to describe the presence and distribution of the so far known species within the genera Pasteurella and Mannheimia in the nasopharynx of cattle. Special reference has been made to the detection method and tetracycline resistance its determinants, since this has often been reported to be present within organisms of the Pasteurellaceae [8] and because tetracyclines are the most common antibiotics used in veterinary medicine [9].
2. MATERIALS AND METHODS

2.1. Animals and Sampling
A total of 57 Holstein, Belgian Blue and mixed breed calves, less than 4 months old and originating from 13 Flemish herds (extensively housed) were examined. On each herd, a maximum of 5 animals without a history of antimicrobial therapy prior to sampling (30 days) were included in the study. Sampling was performed in the period December 2002- March 2003, which is the season with the highest incidence of BRD in the region. Prior to sampling, the nostril was disinfected using alcohol 90%. A sterile swab was introduced in the nasal cavity (dorsal conchae, 15 cm depth) and rotated 360°. After sampling, each swab was inserted into a transport medium (Venturi Transsystem®), Copan and cooled (4-7°C) during transport. Bacteriological investigations were set up within 2 hours after sampling.

2.2. Bacteriological identification
The primary isolation of bacteria was done by directly streaking one side of each swab onto Columbia blood agar containing 5% sheep blood (Oxoid) to which 16 µg/ml bacitracin (Alpha Pharma) was added. Likewise the other side of the swab was streaked onto Columbia blood agar containing 5% sheep blood (Oxoid) to which bacitracin (Alpha Pharma) and oxytetracycline (Sigma) at a concentration of 16 µg/ml and 4 µg/ml, respectively, were added. The plates were used within three days after preparation. Bacteria grown aerobically at 37°C were selected by colonial morphology at 24h and 48h post incubation, followed by subculturing for another 24 h on Columbia blood agar containing 5% sheep blood (Oxoid). If no Pasteurella or Mannheimia species were isolated, the bacteriological investigation was considered negative. Colonies resembling Pasteurella or Mannheimia isolates were checked for purity, morphology was re-evaluated and bacteria were tentatively designated as Pasteurella or Mannheimia [3]. The identification of the organisms up to (sub)species level was molecularly confirmed by means of tDNA-PCR as described earlier [5].

2.3. Antimicrobial resistance analysis
Each identified Pasteurella or Mannheimia organism underwent susceptibility testing through the Kirby Bauer disc diffusion test by means of Neosensitabs® (Rosco) for following antimicrobials: tetracycline, sulphonamide/trimethoprim, ampicillin, amoxicillin+clavulanate, enrofloxacin, and cepfotax. Testing was done according to NCCLS-guidelines with the exception that Columbia agar supplemented with 5% sheep blood was used as medium. E. coli ATCC 25922 was used as reference strain. Bacteria harbouring consistent tetracycline resistance were investigated for the presence of plasmid DNA as described previously [8]. Shortly, plasmids were alkaline lysed, purified by affinity chromatography on Qiafilter® Midi columns (Qiagen), and seperated by agarose gel electroforese. In case of a tet(H) positive isolate and the respective detected plasmids were directly transformed into E. coli TOP10 vectors by the TOPO® transformation procedure (Invitrogen). Tetracycline resistant transformants were selected during overnight incubation by 37°C under aerobic conditions on Luria-Bertani agar (LB) supplemented with 20 mg/ml oxytetracycline (Caesar & Lorenz GmbH). Confirmation of transformation was investigated by reinvestigation of the tetracycline resistance plasmid profiles in the transformant E. coli TOP10 organisms by alkaline lysis followed by agar gel electroforese. In case of a successful transformation experiment, the respective plasmid DNA underwent PCR amplification in order to investigate the presence of tet(H). For plasmid analysis, the bacterial collection was extended with 5 tetracycline resistant Pasteurellaceae (4 Mannheimia (M.) haemolytica and 1 Pasteurella (P.) multocida isolates) isolated from calves suffering from BRD.

3. RESULTS

3.1. Bacteriological investigations
Thirty-five % of the bacteriological investigations were negative (n=20). A total of 40 Pasteurellaceae were isolated in 34 calves (59.6%) out of 10 herds (76.9%). Identification of the strains resulted mainly in Pasteurella (P.) multocida (n=35). Other bacteria were P. canis (1), P. trehalosi (1), Mannheimia (M.) varigena (2) and one untypable Mannheimia spp. Mannheimia haemolytica sensu stricto was not isolated.

3.2. Antimicrobial resistance analysis
Antimicrobial resistance patterns of the strains identified as Pasteurella multocida (n = 35) revealed 100% susceptibility for ampicillin, amoxicillin+clavulanate, enrofloxacin, ceftiofur and the combination sulphonamides+trimethoprim. Five P. multocida isolates, all originating from the same herd, were resistant to tetracycline and 12 P. multocida strains were resistant (n=1) or intermediate susceptible (n=11) for tylosin. The three Mannheimia isolates and the P. trehalosi strain were only resistant for tylosine. The remaining organisms (n=22) were fully susceptible for the tested antimicrobials. In the tetracycline resistant P. multocida isolates from the clinically healthy calves no plasmid DNA was detected.
Of the additional Pasteurellaceae series originated out of pneumatic bovine lungs, two M. haemolytica and one P. multocida were found to contain plasmid DNA. Only from one P. multocida strain the tetracycline resistance was successfully transformed into the E. coli TOP10 acceptor bacteria. Two clones of the transformant E. coli demonstrated a plasmid profile similar to the donorstrain. PCR detection of the tet(H) gene in both the donorstrain and the transformant E. coli revealed the presence of this gene.

4. DISCUSSION
Since the aforementioned substantial reclassification within the Pasteurellaceae, no investigations have been carried out in order to describe the prevalence of these different species in the nasopharynx of calves. However, this knowledge is essential in order to evaluate nasal cultures for diagnostic and epidemiological purposes. Especially since recently a good correlation has been found between the bacteriological identification results of pared nasopharyngeal swabs and bronchoalveolar lavages in the individual pneumatic calf [6]. Based on unpublished preliminary studies, we emphasize the need for disinfection of the nostrils prior to sampling and the use of a selective medium (e.g. Columbia sheep blood agar + 16 μg/ml bacitracine) for a better detection rate of Pasteurellaceae in the nasopharynx. Remarkably, none of the detected Mannheimia strains was identified as M. haemolytica. This is of particular interest because older reports [1] described the presence of [P.] haemolytica in the upper respiratory tract of healthy cattle, and [P.] haemolytica has recently been reclassified into five new Mannheimia species with M. haemolytica being the type species [2].

Antimicrobial resistant strains were rare and in accordance with a previous report on pathogenic Pasteurellaceae out of the same region [3]. The limited use of antimicrobials and the extensive housing conditions in the investigated animals may have contributed to the overall low prevalence of antimicrobial resistant strains found. In the original series Pasteurellaceae, no plasmid DNA was detected in the 5 tetracycline resistant strains. The origin of the decreased tetracycline susceptibility is therefore probably clonally which is indicated by the fact that these strains all were isolated in the same herd. Of the additional Pasteurellaceae strains, one strain proved to harbour a plasmid mediated tetracycline resistance gene. The identified tetH gene, is the most encountered tetracycline resistance gene within the family of Pasteurellaceae [8]. Further investigations are necessary to explore the identity and localisation of the other antibiotic resistance genes involved.

In conclusion, antimicrobial susceptible P. multocida strains were the predominant Pasteurellaceae present in the nasopharynx of young extensively housed calves.

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6. REFERENCES