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Gram-Negative Bacteria from Pharynx and Nasal Cavity of Domestic Goats in Grenada, and Resistance of *Mannheimia haemolytica* and *Bibersteinia trehalosi* to Tulathromycin and Trimethoprim-Sulfamethoxazole

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HH, AR and RNS designed the study and author GS managed collection of all the samples. Authors HH, AR, VMB and TV carried out the laboratory tests. Authors HH and AR wrote the protocols and the first and final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was conducted to evaluate the occurrence of bacterial respiratory pathogens, particularly members of the family *Pasteurellaceae* in healthy domestic goats in Grenada, and to determine the antimicrobial susceptibility of the predominant species.

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Study Design: Nasal and pharyngeal swabs from 161 adult goats from the six parishes of Grenada were collected during a ten month period from May 2012 to March 2013 and examined for potential bacterial respiratory pathogens.
Methodology: Bacteria resembling *Pasteurellaceae*, and *Corynebacterium* spp. were presumptively identified by phenotypic characteristics. For definitive identification to species level, DNA from the isolates were subjected to 16s ribosomal RNA sequencing. The closest matches to sequences in GenBank, and their percentage identity were the criteria used to determine the bacterial species. The major members of *Pasteurellaceae* were tested for antimicrobial susceptibility to 11 antibiotics using the disk diffusion method.
Results: Of a total of 98 Gram-negative isolates, 41% were *Mannheimia haemolytica*, followed *by Bibersteinia trehalosi* (37%), *Mannheimia glucosida* (9%), and the remainder comprising of 11 different species, including five species of *Moraxella*. Of the three Gram-positive isolates, two were *Rhodococcus equi*, and one was *Trueperella pyogenes*. Antimicrobial susceptibility tests on a total of 73 isolates of *M. haemolytica* and *B. trehalosi* showed that 18% isolates were resistant to tulathromycin, a recently introduced drug for use in goats. Moreover, 77% of isolates were resistant to trimethoprim-sulfamethoxazole, another drug with application in goats. Tulathromycin resistance was accompanied by resistance to trimethoprim-sulfamethoxazole in 12 of the 13 isolates. Resistance to these two drugs is not in accordance with published data, and need detailed further investigation. Resistance to ceftiofur, a drug used for pneumonic pasteurellosis was minimal (one isolate only), and none of the isolates were resistant to amoxicillin-clavulanic acid or enrofloxacin.
Conclusion: In conclusion, our study, first of its kind in the Caribbean, showed that *M. haemolytica* and *B. trehalosi*, two major respiratory pathogens of ruminants colonize nasal cavity and pharynx of healthy goats in Grenada. Both organisms showed uncommon high resistance to tulathromycin and trimethoprim-sulfamethoxazole, the reasons for which are not understood, and need further investigation.

Keywords: Mannheimia haemolytica; Bibersteinia trehalosi; goats; nasal; pharynx; antibiotic resistance; Grenada.

1. INTRODUCTION

Small scale sheep and goat farms are common in Grenada, because these animals are more suitable than cattle for the slopped terrain and limited pasture area of this island nation in the southern Caribbean. There is much less published information on bacterial respiratory pathogens of goats compared to sheep. The members of the family *Pasteurellaceae* are generally considered opportunistic secondary invaders which under normal conditions coexist peacefully with the animal host on mucosal membranes of the upper respiratory tracts [1]. There is little information about colonization of different members of this family in the mucosa of goats, particularly with regard to the animals in a tropical country like Grenada. Studies on this aspect are important to understand the epidemiology, particularly because several taxonomic changes have taken place with regard to the family *Pasteurellaceae* [2].

The organism formerly known as *Pasteurella haemolytica* (currently, *Mannheimia haemolytica*) included two biochemical types A and T (indicating arabinose and trehalose fermentation). The T biotype, *Pasteurella trehalosi* has been reclassified as *Bibersteinia trehalosi* [3]. The other species under the genus *Mannheimia*, are: *M. glucosida*, *M. granulomatis*, *M. ruminalis*, and *M.varigena* [4]. Both *M. haemolytica*, and *B. trehalosi* are pathogens of ruminants, and these can be present as commensals of the upper respiratory tracts of domestic and wild ruminants [5]. Both *M. haemolytica* and *B. trehalosi* may be isolated from the lungs of goats with clinical pneumonia [6]. Respiratory viruses dramatically increase the susceptibility of sheep and goats to secondary *M. haemolytica* infection. The infection can be acute resulting in death, but survivors may become chronically affected showing reduced lung capacity and weight gain efficiency [7].

Bacteria associated with pneumonic lesions in goats may include other Gram-negative bacteria, including *Pasteurella multocida*, and *Haemophilus* spp., and Gram-positive bacteria, including *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes* / *Actinomyces pyogenes*) [8,9]. *T. pyogenes* has also been reported to be associated with pharyngitis in sheep and goats, with severe lesions

progressing to abscesses [10]. Atrophic rhinitis associated with toxigenic strains of *Pasteurella multocida* has been recognized in goats. Signs in affected animals include purulent nasal discharge, nose bleeding, sneezing, and occasionally tender or distorted noses. Raised coalescing nodules on the nasal septum and turbinates can occur in goats in tropical countries [9].

Antibiotics used for treatment of bacterial pneumonia in goats include penicillin, ampicillin, tetracycline, and ceftiofur [9]. Tulathromycin has recently been reported to be efficacious for pneumonic infections in goats, and it has been evaluated as a safe drug for goats [11,12].

The objectives of this study were to identify the potential pneumonic bacterial pathogens in the nasal cavity and pharynx of domestic goats in Grenada, and to determine their susceptibility of the predominant isolates to antimicrobial drugs, including tulathromycin.

2. MATERIALS AND METHODS

Between 2012 and 2013, bilateral nasal swabs and pharyngeal swabs (Carry Blair Transport swabs, Becton, Dickinson and Company, Sparks, MD, USA) from a total of 161 goats were collected for bacteriological examination by culture. The goats were adults, and selected randomly from small scale farms from all parishes of Grenada and Cariacou Island, a part of Grenada. The number of animals sampled from each parish was: St. Andrew's, 10; St. David's, 20; St. George's, 28; St. John's, 19; St. Mark's, 25; St. Patrick's, 23; and Cariacou, 36. Samples were stored at 4ºC immediately after collection, and cultured within 24 hours. All samples were plated onto blood agar and MacConkey agar, and incubated aerobically for up to 72 hours. Gram stain was done on different colony types, and subcultures were done from colonies of Gram-negative bacteria resembling members of *Pasteurella, Haemophilus,* and *Moraxella*. Gram-positive bacteria resembling corynebacteria in morphology were also subcultured. Primary identification was based on key phenotypic tests outlined by Markey et al. [13].

For a definitive identification of isolates, bacterial DNA was obtained from a loopful of culture suspended in 200 µl of phosphate buffered saline, boiled for 20 minutes and centrifuged for 3 minutes at 13,000 x g. An aliquot of 20 µl of supernatant was transferred to an FTA card (Whatman Inc., Florham Park, NJ) and mailed to the Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota. Bacterial DNA embedded in FTA cards was eluted and a 500 bp segment of the 16s ribosomal RNA gene was sequenced following previously published protocols [14,15]. The obtained sequences were compared against available sequences in GenBank using the basic local alignment search tool (BLAST) [16] to identify the bacterial species. The closest matches and their percentage identity were the criteria used to determine the bacterial species.

Antimicrobial susceptibility tests were done using the disk diffusion method as recommended by the Clinical and Laboratory Standards Institute [17] and the zone sizes were interpreted as per the modified CLSI guidelines published by Markey et al. [13]. The antibiotic disks used were: amoxicillin-clavulanic acid, ampicillin, ceftiofur, enrofloxacin, gentamicin, lincomycin, neomycin, penicillin, trimethoprim-sulfamethoxazole, tetracycline, and tulathromycin (Becton, Dickinson & Co., Franklin Lakes, NJ).

3. RESULTS

A total of 98 Gram-negative bacterial isolates, recovered from 92 of 161 goats (57%) were identified to species level by 16s ribosomal RNA gene sequencing. Except for 6 isolates, each one was recovered from a different animal (footnote, Table 1). Of the 98 isolates, 40 (41%) were *Mannheimia haemolytica*. The next most common species was *Bibersteinia trehalosi* (37%), followed by *Mannheimia glucosida* (9%). The remaining isolates comprised of 11 different species, each ≤2 in number (Table 1). Based on the 16s ribosomal RNA gene sequence, the mean percent identity for the *M. haemolytica* isolates was 99.34, with a range of 98.6 to 100. For *B. trehalosi*, the mean was 99.01 with a range of 98.0 to 99.7. Six *Moraxella* isolates were recovered with a percent identity range of 97.4 to 99.2, and these included two isolates of *M. bovis*. The other Gram-negative isolates were: *Mannheimia caviae, Acinetobacter* spp., *Enterobacter cloacae, Pantoea stewartii,* and *Bergeyella zoohelcum*, all with a percent identity range of 97.1 to 99.9. Only 3 isolates resembled corynebacteria, initially, and these were confirmed as a *Trueperella pyogenes* isolate, with 100% identity, which was recovered from a pharyngeal swab, and two isolates of *Rhodococcus equi,* with 99.9% identity for both,

recovered from nasal swabs of two different goats.

Antimicrobial susceptibility tests on a total of 73 isolates consisting of 40 *M. haemolytica* and 33 *B. trehalosi* isolates showed no resistance to amoxicillin-clavulanic acid, enrofloxacin, or neomycin (Table 2). Resistance to ampicillin, ceftiofur, or penicillin, was evident among ≤ 4.1% of the isolates. Thirteen isolates (18%) showed resistance to tulathromycin. Resistance to trimethoprim-sulfamethoxazole was 77%. All isolates were resistant to lincomycin. Tulathromycin resistance was accompanied by resistance to trimethoprim-sulfamethoxazole in 12 of the 13 isolates. Two isolates showed additional resistance to tetracycline, and one to ampicillin.

Table 1. Identification based on 16s ribosomal RNA gene sequence of 98[a] gram-negative bacterial isolates from pharynx and nasal cavity of goats (N=92 of 161 animals tested)

Identification of isolate (closest match)	% Identity/range (mean)	No. of isolates (%)	Pharynx	Nasal cavity
Mannheimia haemolytica	98.6-100 (99.3)	40 (40.8)	34	6
Bibersteinia trehalosi	98-99.7 (99)	36 (36.8)	36	0
Mannheimia glucosida	98.8-99.9 (99.2)	9 (9.2)	7	2
Moraxella cuniculi	99-99 (99)	2 (2)	2	0
Moraxella bovis	97.4-97.7 (97.6)	2 (2)	2	0
Moraxella ovis	99	1 (1)	0	1
Moraxella lacunata	97.9	1 (1)	0	1
Moraxella oblonga	99.2	1 (1)	1	0
Mannheimia caviae	98.3	1 (1)	1	0
Acinetobacter bereziniae	99.9	1 (1)	1	0
Acinetobacter indicus	99.5	1 (1)	1	0
Enterobacter cloacae	99.7	1 (1)	1	0
Pantoea stewartii	99.6	1 (1)	1	0
Bergeyella zoohelcum	97.1	1 (1)	0	1
Total	97.1-100 (98.9)	98	87	11

a. Each isolate was from a different animal except for 6 goats, each with 2 bacterial species:
1. *Mannheimia haemolytica, Acinetobacter bereziniae, both from pharynx*
2. *Bibersteinia trehalosi, Enterobacter cloacae, both from pharynx*
3. *Mannheimia haemolytica (nasal cavity), Bibersteinia trehalosi (pharynx)*
4. *Bibersteinia trehalosi (pharynx), Moraxella ovis (nasal cavity)*
5. *Mannheimia haemolytica (nasal cavity), Pantoea stewartii (pharynx)*
6. *Bibersteinia trehalosi (pharynx), Rhodococcus equi (nasal cavity)*

Table 2. Antimicrobial drug resistance among *Mannheimia haemolytica* and *Bibersteinia trehalosi* isolates from throat and nose of goats

Antimicrobial drug	*M. haemolytica*		*B. trehalosi*		Total (N=73)	
	No. resistant	% resistant	No. resistant	% resistant	No. resistant	% resistant
Amoxicillin-clavulanic acid	0/40	0	0/33	0	0	0
Ampicillin	3/40	7.5	0/33	0	3	4.1
Ceftiofur	1/40	2.5	0/33	0	1	1.4
Enrofloxacin	0/40	0	0/33	0	0	0
Gentamicin	0/40	0	1/33	3.03	1	1.4
Lincomycin	40/40	100	33/33	100	100	100
Neomycin	0/40	0	0/33	0	0	0
Penicillin	3/40	7.5	0/33	0	3	4.1
Trimethoprim-sulfamethoxazole	31/40	77.5	25/33	75.8	56	76.7
Tetracycline	1/40	2.5	3/33	9.09	4	5.5
Tulathromycin	7/40	17.5	6/33	18.2	13[a]	17.8

[a] *Resistance patterns (excluding lincomycin) of the 13 tulathromycin resistant isolates: TUL: 1; TUL+TMS: 9; TUL+TMS+TE: 2; TUL+AM+TMS: 1; TUL = Tulathromycin, TMS = Trimethoprim sulfamethoxazole, TE = Tetracycline, AM = Ampicillin*

4. DISCUSSION

Angen et al. [4] and Poulsen et al. [18] used sequencing of the 16S rRNA genes as one of the methods for identification of *Mannheimia haemolytica*-like strains. Determination of 16S rRNA gene sequences is often routinely carried out for *Pasteurellaceae* isolates in many laboratories [2]. However, *M. haemolytica* and *B. trehalosi* are very similar [19], and misclassification can occur, but that would not impact the significance of the results. Both species are potential pathogens of ruminants, and we report antibiotic susceptibility of these predominant organisms with reference to species, and together as a whole.

Pasteurellaceae, consisting of *M. haemolytica*, *B. trehalosi*, and *Pasteurella multocida*, were isolated from nasal and oropharyngeal swabs from both healthy and diseased adult sheep in the United States in a recent study [20]. These bacterial species have also been isolated from pneumonic lungs of goats [6], but published information on carriage of these bacteria in the nasal cavity and pharynx of goats is lacking. It is interesting that 80% of the Gram-negative isolates in the present study comprised of *M. haemolytica* and *B. trehalosi*. Of the 76 isolates of these species, > 90% were recovered from pharynx, and the remaining from the nasal cavity. Our results show that apparently healthy goats in this tropical island can carry these potentially pathogenic organisms in the upper respiratory tracts of goats, similar to limited published findings with regard to goats in the United States [21] and sheep in Norway and the U.S. [18,20]. Nine of isolates in our study were *M. glucosida*, an organism which is potentially pathogenic to small ruminants [22]. In a study in Norway, a relatively high number of apparently healthy sheep were found to carry *M*. *glucosida*, in addition to *M. haemolytica* in their nose [18]. Of the 5 species of *Moraxella* isolated in the present study, except for *M. bovis*, a known eye pathogen of cattle, and a respiratory pathogen of a chamois, a goat-antelope [23], the significance of various species in animals is not well understood. Most of them are found as part of the indigenous microbiota of animals, including goats [13,24]. *Mannheimia caviae* has been isolated from epidemic conjunctivitis and otitis media in guinea pigs [25]. We did not look for *Acinetobacter* spp. or *Enterobacteriaceae*. However, two isolates resembling *Moraxella*, turned out to be *Acinetobacter* spp. *Acinetobacter bereziniae* has been isolated from environment, including sewage, and human and animal specimens [26]. Other *Acinetobacter* spp. including *A. indicus*, one of the isolates in the present study, can be found in the environment, and dump sites [27]. *Enterobacter cloacae* may be found in water, soil, and sewage [28], and is of no significance in this study. *Pantoea stewartii,* a phytopathogen, has been isolated from mosquitoes and water from their breeding areas [29,30]. *Bergeyella zoohelcum* is a zoonotic pathogen found in the upper respiratory tracts of dogs and cats, and it can cause cellulitis, abscesses, bacteremia, and pneumonia in humans from animal bites [31]. A case of bacteremia following ingestion of a dish prepared with goat blood has been reported [32]. Only 3 isolates in this study had coryneform morphology, and among these one was *Trueperella pyogenes*, an organism associated primarily with ruminants, including goats [33]. The isolate was recovered from the pharynx of a goat*. T. pyogenes* has been reported to be associated with pharyngitis in sheep and goats, with severe lesions progressing to abscesses [10]. The other two isolates were *Rhodococcus equi*, mainly, a horse pathogen, recovered from nasal swabs of two different goats. This organism has been reported to cause abscesses in liver, lung, and fatal disseminated infection in goats [34-36]. The epidemiology of this equine pathogen is not well understood, but it is interesting that two healthy goats in our study were carriers of this organism in the nasal cavity.

In the present study, only a small number of isolates (<10%) were resistant to ampicillin, ceftiofur, penicillin or tetrcycline*.* In a study in California on 36 respiratory tract isolates, mainly consisting of *Mannheimia haemolytica* and *Pasteurella multocida* of caprine origin, Berge et al. [37] noted that all were susceptible to ceftiofur. In their study, more than 95% of the isolates were susceptible to tetracycline as well. Antimicrobial resistance was not found to be a problem. In a South African study, a long-acting doxycycline preparation was found to be effective in a goat model of *P (M). haemolytica* pneumonia [38]. Goat isolates of *M. haemolytica* and *B. trehalosi* from the United States were found to be susceptible to commonly used antibiotics. In addition, recently introduced antibiotic, tulathromycin, a triamilide macrolide, has been identified as a potentially useful drug in goats [6]. In our study, nearly 18% of isolates of these species were found resistant to tulathromycin, and resistance to this drug was accompanied by resistance to trimethoprim-sulfamethoxazole in

12 of the 13 isolates. The resistance rate of 77% of isolates to trimethoprim-sulfamethoxazole in the present study is not in accordance with published data [39], which suggests a susceptibility rate of >96%. The reason for the uncommon resistance rates to tulathromycin and trimethoprim-sulfamethoxazole among the isolates from goats in this island nation needs further investigation. The use of these drugs is none or minimal in Grenada; a possible reason may be the unique nature of the strains adapted to goats in this tropical nation. A limitation of this study is that we did not determine the minimal inhibitory concentrations (MICs) of the drugs. Future studies should include determination of MICs as well. In the present study, lincomycin was included, and all isolates of *M. haemolytica* and *B. trehalosi* showed resistance, confirming the lack of efficacy of this drug on "*Pasteurella* group" of bacteria [40].

5. CONCLUSION

Our study, first of its kind in the Caribbean, showed that *Mannheimia haemolytica* and *Bibersteinia trehalosi*, two major respiratory pathogens of ruminants colonize nasal cavity and pharynx of considerable number of healthy goats in the tropical country, Grenada. In addition, other members of the genus *Mannheimia*, including *M. glucosida, M. caviae*, and *Moraxella* spp., including *M. bovis*, were identified, and confirmed by DNA sequencing. *Rhodococcus equi*, primarily an equine pathogen was isolated from two goats. Antimicrobial susceptibility tests for *M. haemolytica* and *B. trehalosi* showed unusual high resistance to tulathromycin and trimethoprim-sulfamethoxazole, two drugs with application in goats. All but one of the tulathromycin resistant isolates showed additional resistance to trimethoprim-sulfamethoxazole, and further studies are required to analyze this uncommon pattern. Resistance was minimal or none to amoxicillin-clavulanic acid, ceftiofur, and enrofloxacin, which agrees with the published information from other countries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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