Veterinary Microbiology 187 (2016) 75–81

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Evolution of the nasopharyngeal microbiota of beef cattle from weaning to 40 days after arrival at a feedlot

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ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form 15 March 2016
Accepted 25 March 2016

Keywords:
Bovine respiratory disease
Metagenomics
16S rRNA gene
Shipping fever

ABSTRACT

Bovine respiratory disease complex (BRDc) is a major cause of morbidity and mortality in beef cattle. There is recent evidence suggesting that the nasopharyngeal microbiota has a key role in respiratory health and disease susceptibility in cattle. However, there is a paucity of knowledge regarding evolution of the nasopharyngeal microbiota when cattle are most likely to develop BRDc (*i.e.*, from weaning to 40 days after arrival at a feedlot). The objective was to describe the evolution of the nasopharyngeal microbiota of beef cattle from weaning to 40 days after arrival at a feedlot. Deep nasal swabs (DNS) from 30 Angus-cross steers were collected at weaning, on arrival at a feedlot, and at day 40 after arrival. The DNA was extracted from DNS and the hypervariable region V3 of the 16S rRNA gene was amplified and sequenced (Illumina MiSeq platform). Nasopharyngeal microbiota underwent a profound evolution from weaning to arrival at the feedlot and from arrival to day 40, with the abundance of 92 Operational Taxonomic Units (OTUs) significantly changing over time. *Mycoplasma* (*M. dispar* and *M. bovirhinis*) was the most abundant genus in the nasopharynx, accounting for 53% of the total bacterial population. Because an evolving bacterial community may be less capable of resisting colonization by pathogenic bacteria, the instability of the nasopharyngeal microbiota documented in this study might explain why cattle are most likely to be affected with BRDc during the first weeks after weaning and arrival at a feedlot.

1. Introduction

Bovine Respiratory Disease Complex (BRDc) is one of the most important health problems in the beef industry (USDA, 2013). Beef cattle of all ages can be affected with BRDc; however, they are most likely to be affected during the 40 days after entrance into the feedlot because they are exposed to a wide range of pathogens (due to commingling) concurrent with various stressors (*e.g.*, weaning and transportation), which can suppress their immune system (Taylor et al., 2010).

Important pathogenic bacteria associated with BRDc include *Mannheimia haemolytica*,*Pasteurella multocida, Histophilus somni* and *Mycoplasma bovis* (Griffin et al., 2010). These bacteria can reside in the upper respiratory tract (URT) of healthy cattle as commensal organisms. However, under specific conditions (*e.g.*, stress and viral infections), they can proliferate in the URT and gain access to the lungs through inhalation (Caswell, 2014). Whereas host immunity is important in controlling overgrowth of pathogens in the URT, there is recent evidence that the composition of the nasopharyngeal microbiota has a crucial role in determining respiratory health in ruminants (Holman et al., 2015a). Unfortunately, because most studies have focused only on BRDc pathogens, there is a paucity of information concerning the overall composition of nasopharyngeal microbiota of cattle and its role in maintaining health. Notwithstanding, such information is crucial to better understand the pathogenesis of BRDc and thus improve its prevention, diagnosis and treatment.

The objective was to describe the composition of the nasopharyngeal microbiota of beef cattle and its evolution from weaning to 40 days after arrival at a feedlot (when these animals are most likely to be affected with BRDc).

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http://dx.doi.org/10.1016/j.vetmic.2016.03.020
0378-1135/

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care (Olfert et al., 1993). The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC13-0104).

2.2. Animals and sample collection

Thirty Angus beef steers (initial body weight 258 ± 27 kg) were studied from weaning to 40 days after entrance into a research feedlot (Agriculture and Agri-Food Canada Research Centre, Lethbridge, AB, Canada), between November 2013 and December 2013. All steers originated from the same cow-calf ranch (Onefour Research Ranch, SW15 T2 R4, Canada). They had been dehorned and castrated at least 45 days before weaning and vaccinated against *M. haemolytica* and *H. somni* (Somnu-Star Ph; Novartis, Mississauga, ON, Canada), bovine herpes virus 1 (BoHV-1), bovine viral diarrhea virus (BVDV) Types 1 and 2, parainfluenza 3 virus (Pi-3), and bovine respiratory syncytial virus (BRSV) (Express 5; Boehringer Ingelheim, Burlington, ON, Canada), and *Clostridium chauvoei, C. haemolyticum, C. novyi* Type B, *C. perfringens* Types B, C and D, *C. septicum and C. tetani* (Tasvax 8; Merck Animal Health, Kirkland, QC, Canada). Primary and booster vaccinations were administered at 48 ± 21 and 129 ± 21 days of age, respectively.

At weaning on the cow-calf ranch (day 0; 196 ± 21 days of age), steers were separated from their dams and immediately sampled using guarded deep nasopharyngeal swabs (DNS). Thereafter, calves were transported for 2 h to a commercial auction market located in Southern Alberta, held overnight in contact with other cattle, and then transported for a duration of 8 h before delivery to the research feedlot. This protocol aimed at simulating transportation and marketing that is typical for calves placed in commercial feedlots in Western Canada. Upon arrival at the research feedlot, calves were offloaded and allowed to rest overnight in a pen with *ad libitum* access to hay and water.

The next morning (day 2), steers were re-sampled by DNS, dewormed, identified using a visual ear-tag and weighed. They did not receive any vaccine or antibiotic to prevent BRDc. Afterwards, cattle were housed in two outdoor dirt-floor pens with straw for bedding. They had *ad libitum* access to fresh water and were fed once daily at 0900 h a total mixed ration (70% barley silage, 25% barley grain, and 5% mineral and vitamin supplement; dry matter basis) that met or exceeded National Research Council Recommendations (NRC, 2000). This ration did not change during the study period (*i.e.*, no transition).

Steers were observed twice daily for clinical illness. Steers with visual signs of BRDc such as depression, decreased rumen fill compared to pen-mates, nasal or ocular discharge, cough, and increased respiratory rate (>40 breaths/min) were removed from the pen and examined. Steers with visual BRDc signs and rectal temperature ≥40.0 °C were defined as a BRDc case and treated with florfenicol and flunixin-meglumine (Resflor, Merck, Kirkland, QC, Canada). At the end of the study period (*i.e.*, day 42), steers were re-sampled by DNS and weighed.

2.3. Sampling procedures

Deep nasal swabs were collected as described (Timsit et al., 2013) using long guarded swabs (27 cm) with a rayon bud (MW 124, Medical Wire & Equipment, Corsham, UK). Two DNS were collected per animal (one swab per nasal cavity). Immediately after collection, DNS samples were placed into Amies transport media, transported in a container (temperature, 4 °C) and processed within 6 h after collection. During this processing, rayon tips were removed from DNS and placed back into the Amies media, followed by centrifugation (13,000*g* for 5 min) to pellet the bacteria and tip. Afterwards, resulting pellets were stored at −80 °C pending DNA extraction.

2.4. DNA extraction

Total DNA was extracted from pellets using a DNeasy Tissue kit (Qiagen Inc., Mississauga, ON, Canada) as described (Holman et al., 2015b). Briefly, pellets were thawed at 4 °C and re-suspended in 180 μl of enzymatic buffer that contained mutanolysin (300 U ml^{-1}) in addition to lysozyme (20 mg ml^{-1}). The mixtures were vortexed and then incubated for 1 h at 37 °C. Then, 25 μl of proteinase K and 200 μl Buffer AL (without ethanol) were added, followed by vortexing and incubation at 56 °C for 30 min. Approximately 600 mg of 0.1 mm zircon/silica beads were added and mixed using a Tissue Lyser II (Qiagen) at maximum amplitude for 3 min. These mixtures were then centrifuged (13,000*g* for 5 min), and 200 μl of ethanol was added to the supernatants, followed by vortexing. Thereafter, the protocol of the DNEasy Tissue Kit was followed in accordance with manufacturer's instructions.

2.5. Amplification and sequencing of bacterial 16S rRNA gene

The V3 region of the 16S rRNA gene was amplified using modified 341F and 518R primers, as described (Bartram et al., 2011). Three PCR amplifications were done for each sample, using 50-μl reaction mixtures. Each reaction mixture contained 25 pmol of each primer, a 10 mM concentration of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, and 1 U Taq polymerase. The PCR conditions involved an initial denaturation step at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s and ended with an extension step at 72 °C for 10 min in a S1000 thermal cycler (Bio-Rad, Mississauga, ON, Canada). Following separation of products from primers and primer dimers by electrophoresis on a 2% agarose gel, PCR products of the appropriate size (*i.e.*, 300 bp) were recovered using a Purelink Gel Kit (Thermo Fisher Scientific Inc., Ontario, Canada). The 16S rRNA gene amplicons were then sequenced using the MiSeq reagent kit v2 (500 cycles) on the MiSeq platform (Illumina, Inc., San Diego, CA, USA) according to manufacturer's instructions.

2.6. Sequence and statistical analyses

Data were processed using a custom Snakemake pipeline (https://bitbucket.org/mlworken/microbiome-pipeline; Köster and Rahmann, 2012) based on the UPARSE pipeline (Edgar, 2013) using USEARCH v8.0.1623. Briefly, for each set of forward and reverse reads, the forward and reverse 16S primers were removed with cutadapt v1.8 (Marcel, 2011). Paired reads were merged with the USEARCH fastq_mergepairs option. Following quality assessment, merged reads were quality-filtered using the maximum expected error method in USEARCH (cutoff of 0.5 and reads truncated to 175 bp). Processed reads from each sample were combined into a single file which was then de-replicated and singletons were removed with VSEARCH v1.1.3 (https://github.com/torognes/vsearch). Sequences were clustered into Operational Taxonomic Units (OTUs) at 97% similarity using USEARCH and the cluster_otus command. The OTUs were re-checked for chimeras with the uchime_ref command in USEARCH. Taxonomy was assigned to each OTU using the RDP