The Veterinary Journal 234 (2018) 1–6

Contents lists available at ScienceDirect

The Veterinary Journal

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

Original Article

Characterisation of *Bergeyella* spp. isolated from the nasal cavities of piglets

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ARTICLE INFO

Article history:
Accepted 20 January 2018

Keywords:
Bergeyella spp
Colonisation
Nasal microbiota
Porcine
Virulence assays

ABSTRACT

The aim of this study was to characterise bacteria in the genus *Bergeyella* isolated from the nasal passages of healthy piglets. Nasal swabs from 3 to 4 week-old piglets from eight commercial domestic pig farms and one wild boar farm were cultured under aerobic conditions. Twenty-nine *Bergeyella* spp. isolates were identified by partial 16S rRNA gene sequencing and 11 genotypes were discriminated by enterobacterial repetitive intergenic consensus (ERIC)-PCR. *Bergeyella zoohelcum* and *Bergeyella porcorum* were identified within the 11 genotypes. *Bergeyella* spp. isolates exhibited resistance to serum complement and phagocytosis, poor capacity to form biofilms and were able to adhere to epithelial cells. Maneval staining was consistent with the presence of a capsule. Multiple drug resistance (resistance to three or more classes of antimicrobial agents) was present in 9/11 genotypes, including one genotype isolated from wild boar with no history of antimicrobial use. In conclusion, *Bergeyella* spp. isolates from the nasal cavities of piglets showed some in vitro features indicative of a potential for virulence. Further studies are necessary to identify the role of *Bergeyella* spp. in disease and within the nasal microbiota of pigs.

Introduction

The microbiota is composed of microorganisms that colonise the skin and mucosal surfaces and lumina, including the alimentary, respiratory and urogenital tracts (Budden et al., 2017). These organisms influence the development and maturation of the innate and adaptive immune responses (Günther et al., 2016). The microbiota can protect against colonisation by pathogens through direct inhibition of the growth of pathogens, modification of the microenvironment or competition for colonisation sites. Conversely, some microorganisms in the microbiota are able to cause disease under particular conditions (Reid et al., 2011).

Respiratory tract infections are among the leading cause of disease and death in human beings and animals throughout the world (Ayrle et al., 2016).[1] Respiratory disease in pigs is often polymicrobial and is influenced by adverse environmental conditions (Brockmeier et al., 2002). Some agents, such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV) and *Actinobacillus pleuropneumoniae*, can act as primary pathogens. Primary infections frequently are complicated by opportunistic bacteria, such as *Pasteurella multocida, Haemophilus parasuis* and *Streptococcus suis*, resulting in more severe respiratory disease (Brockmeier et al., 2002). The relationship between the components of the nasal microbiota, especially carriage of potential pathogens, and respiratory disease in pigs was reported by Bertschinger and Nicod (1970). A deeper knowledge of the composition of the microbiota has been achieved with the introduction of massive sequencing (Slifierz et al., 2015; Correa-Fiz et al., 2016; Niederwerder et al., 2016).

Differences in the composition and diversity of the porcine microbiota act as contributing factors to disease (Correa-Fiz et al., 2016; Niederwerder et al., 2016). Specifically, the nasal microbiota has been shown to influence the development of disease by *H. parasuis* (Correa-Fiz et al., 2016). In the study by Correa-Fiz et al. (2016), the phylum *Bacteroidetes* was abundant in the nasal microbiota of healthy piglets and *Weeksella* was one of the most abundant genera. Holmes et al. (1986) defined two species of *Weeksella* (*Weeksella virosa* and *Weeksella zoohelcum*); *W. zoohelcum* has been renamed *Bergeyella zoohelcum* (Vandamme et al., 1994).

Bacteria from the phylum *Bacteroidetes* that have been used as probiotics include *Prevotella bryantii* in cattle (Chiquette et al., 2012) and *Barnesiella* spp. in human beings (Ubeda et al., 2013).

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[1] See: http://www.who.int/gho/mortality_burden_disease/en (accessed 12 December 2017).

https://doi.org/10.1016/j.tvjl.2018.01.004

Table 1
Bergeyella spp. isolates obtained from nasal swabs of piglets at weaning (10 piglets from each farm were sampled for this study).

Farm	Number of *Bergeyella* isolates	Country	Health status	Antibiotic treatment[a]
AR	4	Spain	Healthy	Amoxicillin
CR	6	Spain	Healthy	Amoxicillin
EJ	2	Spain	Glässer's disease	Enrofloxacin
GM	8	Spain	Healthy	Amoxicillin
GW	1	UK	Healthy	Amoxicillin
LG	4	UK	Healthy	None
LL	1	Spain	Nervous signs	Unknown
MT	2	Spain	Glässer's disease	Penicillin + Streptomycin
WB[b]	1	Spain	Healthy	None

[a] Antibiotic treatment in the perinatal period.
[b] European wild boars. Other farms have domestic pigs.

Although *Bergeyella* spp. are abundant in the nasal microbiota of piglets, the role of these bacteria in health or disease is poorly characterised. In this study, we determined virulence traits and antimicrobial susceptibilities of *Bergeyella* spp. isolated from the nasal cavities of piglets at weaning.

Materials and methods

Sampling and bacterial isolation

Procedures involving animals followed European Union (EU) Directive 2010/63/EU. Samples were collected from healthy piglets, 3–4 weeks of age, from farms with a range of health statuses (Table 1). In some cases, disease was observed in older animals on the farms at the time of sampling, but piglets were unaffected. Nasal swabs from sampled piglets were transported in Amies medium (Deltalab) to the laboratory and were processed for bacterial isolation. Nasal swabs were plated on chocolate agar (Biomerieux) to isolate colonies. After 24 or 48 h incubation at 37 °C in 5% CO_2, colonies were selected and subcultured on chocolate agar to obtain pure cultures for further analysis.

Bacterial identification and genotyping

DNA from each isolate was purified from bacterial suspensions using a Chelex-based Instagene Matrix (Bio-Rad). For preliminary identification, a small fragment of the 16S rRNA gene was amplified and sequenced. This fragment was amplified using oligonucleotide primers 358F (5′-CTACGGGAGGCAGCAGT-3′) and 907R (5′-CCGTCWATTCMTTTGAGTTT-3′) (Lane, 1991). The PCR reaction solution comprised 1.5 mM $MgCl_2$ (Promega), 0.2 mM deoxynucleotide triphosphates (dNTPs; Promega), 0.4 μM each oligonucleotide primer, 3 μL bacterial DNA and 0.75 U GoTaq DNA polymerase (Promega) in a final volume of 25 μL. The amplification cycle was 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, then 72 °C for 7 min (GeneAmp PCR System 2700; Applied Biosystems). After confirming specific amplification by agarose electrophoresis, the amplicons were sequenced with primer 907R. Sequences were analysed using the Ribosomal database Seqmatch[2] application to identify the bacterial genus or species.

Different genotypes of *Bergeyella* spp. were differentiated by enterobacterial repetitive intergenic consensus (ERIC)-PCR (Versalovic et al., 1991) using oligonucleotide primers ERIC-1F (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2R (5′-AAGTAAGTGACTGGGGTGAGCG-3′). DNA concentrations were determined using a μLite (Biodrop). The reaction solution comprised 3 mM $MgCl_2$ (Promega), 1.2 μM each oligonucleotide primer, 0.23 mM dNTPs (Promega), 0.75 U GoTaq polymerase (Promega) and 25 ng DNA in a final volume of 25 μL. The amplification cycle was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2.5 min, then 72 °C for 20 min (GeneAmp PCR System 2700; Applied Biosciences). PCR products (5 μL) were separated by electrophoresis at 80 V for 1 h in 2% agarose gels, stained with ethidium bromide and examined using an ultraviolet transilluminator (U:Genius; Syngene).

Final identification of selected isolates was performed by 16S rRNA gene sequencing using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The PCR reaction solution comprised 2 mM $MgCl_2$ (Promega), 0.4 μM each oligonucleotide primer, 0.2 mM dNTPs (Promega), 0.75 U GoTaq polymerase (Promega) and 250 ng DNA in a final volume of 25 μL. The amplification cycle was 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, then 72 °C for 7 min (GeneAmp PCR System 2700 thermocycler; Applied Biosciences). Amplicons were sequenced with primers 8F, 358F, 907R and 1492R. Sequences were analysed using Fingerprinting II v3.0 software (Bio-Rad) and an unweighted pair group method with arithmetic mean (UPGMA) tree was constructed.

[2] http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp (accessed 12 June 2017).

Antimicrobial susceptibility

Susceptibility to gentamicin, erythromycin, enrofloxacin, tetracycline, doxycycline, trimethoprim-sulphonamide, amoxicillin, ceftiofur, amoxicillin-clavulanic acid and lincomycin-spectinomycin were tested using Neo-Sensitabs (Rosco Diagnostica) by measuring the diameter of growth inhibition. Diameters of the zones of inhibition were measured using a calliper and were compared with the manufacturer's breakpoint tables. Susceptibility to marbofloxacin, florfenicol and tulathromycin were tested by dilution in 96-well plates to determine minimum inhibitory concentrations (MICs). Plates were incubated overnight at 37 °C in 5% CO_2 and the bacterial growth was measured by determining the optical density at 600 nm (OD_{600}) using a spectrophotometer (VIS 7200; Dinko Instruments). Isolates with values greater than or less than the diameter or concentration of the breakpoint suggested by the manufacturer were designated 'sensitive' and 'resistant', respectively.

Biofilm formation

Bergeyella spp. isolates were cultured in 96-well plates (Bello-Orti et al., 2014) that were uncoated or coated with 5 μg/well mucin or bovine serum albumin (BSA) (Sigma–Aldrich). Bacterial suspensions were prepared in brain heart infusion (BHI; Merck) broth to an OD_{600} of 0.3. A 1:10 dilution of the bacterial suspension was made in BHI broth and 100 μL were dispensed in 96-well plates in quadruplicate. Plates were incubated at 37 °C in static conditions or by shaking at 100 revolutions per min. After the appropriate time of incubation, unattached bacteria were removed by vacuum suction and wells were washed once by immersion in water. Biofilms were stained with 0.1% crystal violet for 2 min. Stained biofilms were washed three times with water and then placed at 37 °C until the plates were dry. The dye was released from the biofilm with 100 μL 70% ethanol and quantified by determining the OD_{590} using a spectrophotometer (Powerwave XS Microplate; Biotek Instruments).

Cell adhesion assay

The cell adhesion assay was performed using lung adenocarcinoma A549 cells. Cells were plated in 96-well plates at a density of 500,000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% foetal bovine serum (FBS; Euroclone) and 1% glutamine (Gibco). Cells were grown overnight at 37 °C in 5% CO_2 to form monolayers. Bacterial suspensions of selected isolates of *Bergeyella* spp. were prepared in phosphate buffered saline (PBS) to an OD_{600} of 0.3. Monolayers of A549 cells in 96-well plates were washed once with sterile PBS and inoculated into duplicate wells with 100 μL bacterial suspension, equivalent to ~10^9 colony forming units (CFUs)/mL. Plates were centrifuged for 10 min at 100 *g* to facilitate contact between bacteria and cells, then incubated for 1 h at 37 °C in 5% CO_2. After incubation, wells were washed twice with sterile PBS to remove unattached bacteria. To release the attached bacteria, wells were treated with 0.1% saponine (Sigma–Aldrich) in PBS followed by pipetting. Bacteria in the wells were quantified by dilution and colony counts.

Serum susceptibility assay

Serum susceptibility assays were performed using rabbit serum (Cerdà-Cuéllar and Aragon, 2008); bacteria (10^7–10^8 CFUs/mL) were incubated with 80% serum for 1 h at 37 °C. Bacterial counts were performed by dilution and plating before and after incubation with serum. The logarithmic reduction of bacterial counts after 1 h with serum was then calculated. Selected isolates were also tested with porcine serum using the same conditions.

Phagocytosis assay

Phagocytosis assays were carried out with porcine alveolar macrophages (PAMs) previously harvested from two piglets euthanased by intravenous sodium pentobarbital overdose following EU Directive 2010/63/EU (Olvera et al., 2009).