Variability of *Pasteurella multocida* isolated from Icelandic sheep and detection of the toxA gene

Thorbjorg Einarsdottir,1,2 Eggert Gunnarsson,1 Olof G. Sigurdardottir,1,2 Einar Jorundsson,1,2 Vala Fridriksdottir,1 Gudridur E. Thorarinsdottir3 and Sigridur Hjartardottir4

1Institute for Experimental Pathology, University of Iceland, Keldur, Keldnavegur 3, 112 Reykjavik, Iceland
2BioMedical Center, University of Iceland, Reykjavik, Iceland
3Veterinary Center, Dynskalar 30, 850 Hella, Iceland

*Pasteurella multocida* can be part of the upper respiratory flora of animals, but under conditions of stress or immunocompromise, the bacteria can cause severe respiratory symptoms. In this study, we compared 10 *P. multocida* isolates from Icelandic sheep with respiratory symptoms and 19 isolates from apparently healthy abattoir sheep. We examined capsule type, genetic variability and the presence of the toxA gene in the two groups. Surprisingly, we found that all ovine *P. multocida* isolates examined in this study carried the toxA gene, which markedly differs from what has been published from other studies. Interestingly, all isolates from abattoir animals were capsule type D, whilst bacteria isolated from animals with clinical respiratory symptoms had capsule type A, D or F. Examination of seven housekeeping genes indicated that the clinical respiratory isolates were significantly more heterogeneous than the abattoir isolates (P<0.05, two-tailed Mann–Whitney U test). The results suggest that there may be at least two groups of *P. multocida* in sheep – a genetically homogeneous group that resides in the respiratory tract and a genetically heterogeneous group that is the predominant cause of disease.

INTRODUCTION

*Pasteurella multocida* is a pervasive bacterium that can be part of the normal upper respiratory tract flora of many animal species (Dabo et al., 2007; Weber et al., 1984). The bacterium can cause mild chronic upper respiratory tract inflammation, acute pneumonia or septicaemia (Wilson & Ho, 2013). There are five serotypes of *P. multocida*, based on the composition of the polysaccharide capsule (A, B, D, E and F) (Hatfaludi et al., 2010; Townsend et al., 2001). In addition, untypable strains have been described, which may be acapsular (Bethe et al., 2009; Boyce et al., 2000; Davies et al., 2003; Ewers et al., 2006). The capsule likely serves a function in protecting the bacteria from desiccation, phagocytosis and bactericidal complement activity, and capsular bacteria are more virulent than acapsular strains (Boyce et al., 2000). The capsule type appears to be related to disease predilection; e.g. capsule types B and E correlate with haemorrhagic septicaemia (Boyce et al., 2000; DeAngelis et al., 2002). Capsule typing of *P. multocida* strains indicates that capsule types A and D are the most prevalent in sheep (Prabhakar et al., 2010; Tahamtan et al., 2014; Zamri-Saad et al., 1996).

Another virulence factor is the dermonecrotic toxin (*P. multocida* toxin, PMT). The gene for this toxin, toxA, is located on a lysogenic bacteriophage and appears to be associated with upper respiratory tract disease (Davies et al., 2003). Analysis of six *P. multocida* genomes identified a unique 18 kbp region which contains 14 genes, including the toxA gene, in a porcine strain (Liu et al., 2012; Wilson & Ho, 2013). PMT has primarily been studied in porcine *P. multocida* strains (Kubatzky et al., 2013; Orth & Aktories, 2010; Pullinger et al., 2004; Siegert et al., 2013), although toxigenic bacteria have also been described from other animal species, such as sheep, goats, rabbits and humans (Donnio et al., 1991; Jarvinen et al., 1998; Shayegh et al., 2008). It is not known whether the other 13 genes identified in the whole genomic comparison are present in these non-porcine strains. PMT administration can cause porcine atrophic rhinitis, which is characterized by shortening and twisting of the snout (Pullinger et al., 2004; Wilson & Ho, 2013). The toxin has been shown to experimentally induce a variety of lesions in other animal species, including...
pneumonia, dermal necrosis, osteolysis and hepatic toxicity (Cheville & Rimler, 1989; Chrisp & Foged, 1991; Sterner-Kock et al., 1995), although it is unclear what role the toxin plays in natural disease progression. Shayegh et al. (2008, 2010) described toxigenic *P. multocida* in Iranian sheep and goats and found a higher prevalence of toxigenic bacteria in sheep with pasteurellosis (72.2% of *P. multocida* isolated from 36 animals) compared to bacteria isolated from healthy animals (toxin not detected in *P. multocida* isolated from 11 animals).

Whilst there are very few goats in Iceland, sheep farming is important with a population of approximately 500 000 animals (http://www.statice.is - livestock by regions 2010). The country was divided into 36 quarantine zones in 1941, in order to prevent the spread of the maedi-visna virus. Whilst the virus was eliminated from Icelandic flocks in 1965, the quarantine zones, now 26, remain in order to prevent the spread of other contagious sheep and goat diseases (Fridriksdottir et al., 2003; Guðnadóttir et al., 2013; Pálsson, 1972). Sheep from different zones are thus separated by geographical and man-made barriers that prevent contact between them.

The aim of the current study was to compare the variability and toxigenicity of *P. multocida* isolated from sheep with respiratory symptoms with isolates from apparently healthy abattoir sheep. The findings indicate that whilst all the *P. multocida* isolates examined from Icelandic sheep carried the *toxA* gene, isolates inhabiting the respiratory tract of apparently healthy slaughter animals were more genetically homogeneous than those isolated from sheep with respiratory symptoms.

### METHODS

#### Animals

**Abattoir sheep.** *P. multocida* was grown from abattoir sheep lung samples in October 2013 (referred to as ovine abattoir isolates). The animals were apparently healthy slaughter animals, approximately 6 months old, but had pneumatic lesions compatible with mycoplasma infection upon organ inspection. The diagnosis was confirmed by identifying typical histopathological lesions and by immunohistochemical staining for *Mycoplasma ovipneumoniae*. The animals were brought to the abattoirs from seven farms in different quarantine zones all around the country. For each farm, two to four *P. multocida* isolates were collected from one animal, except for one farm where bacteria were collected from two animals (farm referred to as A2). All isolates from each farm are grouped and referred to as A1–A7, and isolates are distinguished with letters, e.g. A1a and A1b.

**Sheep with respiratory symptoms (referred to as clinical ovine isolates).** *P. multocida* was grown from lung and/or laryngeal samples from sheep with respiratory symptoms in 2003–2012; the diagnoses included atypical pneumonia (*M. ovipneumoniae* infection), pasteurellosis and laryngitis. The animals ranged from lambs to adult animals and came from 10 farms, spread all over the country, with one isolate per farm. The isolates are referred to as C1–C10.

**Other animals.** *P. multocida* was cultured from organs or swabs from animals other than sheep that displayed varied clinical symptoms in 1998–2012. The organ samples included liver, spleen, ovaries and respiratory tract. The samples were from different areas of the country.

### Bacterial isolation and verification

Nineteen abattoir *P. multocida* isolates, 10 clinical ovine isolates and 28 isolates from animals other than sheep were used in this study. Abattoir bacteria were isolated by direct plating from tissue onto sheep blood agar plates (Oxoid CM 55 and defibrinated sheep blood) and incubation under aerobic conditions at 37 °C for 24 h. Confirmation and typing were done by macroscopic and microscopic observations, biochemical testing (Quinn, 1994) and PCR (Deressa et al., 2010). Bacteria from sheep with clinical respiratory symptoms and other animal species were retrieved from the bacterial collection at the Institute for Experimental Pathology at Keldur. For all bacteria, DNA was isolated using NucleoSpin Tissue columns (Macherey Nagel), according to manufacturer’s instructions. The *P. multocida*-specific *kmt1* gene product was amplified in a PTC-200 Peltier Thermal Cycler (MJ Research) using 100 ng DNA with 2 µM KMT1T7 and KMT1SP6 primers (TAG Copenhagen). Illustra PuriTeQ Ready-To-Go PCR Beads (GE Healthcare) were used for all PCRs in this study. The reaction was performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, and then elongation at 72 °C for 5 min. The 460 bp product was electrophoresed in a 2% agarose gel (AppliChem) in a BioRad electrophoresis tank (BioRad) and imaged using an InGenius Bio-imaging system (Syngene). All isolates in this study were positive for the *P. multocida*-specific *kmt1* gene (data not shown).

### Capsule analysis

The *P. multocida* capsule type was analysed using multiplex PCR for genes involved in capsule biosynthesis, as described by Townsend et al. (2001). The product was run on a 0.5% agarose gel with a 2-log DNA ladder (New England BioLabs).

### Toxin analysis

The *toxA* gene was amplified using semi-nested PCR (Choi & Chae, 2001), using 300 ng DNA and 2 µM TA-1 (5'-CTTAGTACGCAGCACAAGG-3', nt 2096±2113) and TA-2 (5'-GAATGCCACACTCTTATAAGC-3', nt 2942±2950) primers for the primary reaction (865 bp product; Fig. 1a). Two micromolar TA-3 (5'-TACAAAGGTCTGGTGCCG-3', nt 2270±2289) and TA-2 primers were used for the secondary reaction (691 bp product; Fig. 1b), using 1 µl of the primary PCR product. Both reactions were performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and then elongation at 72 °C for 5 min.

### Cloning of *toxA* gene segments

The *toxA* gene from abattoir lung samples was cloned into pBAD Topo TA Expression vectors, according to the manufacturer’s instructions (Thermo Fisher Scientific). A segment containing the N and C1 domains (2157 bp, Fig. 1c) was amplified using NF (5'-CCACGATT-CATGAAAACAAAACAT-3', nt 3840±3858) and TA-1 (5'-GAATGCCACACTCTTATAAGC-3', nt 5808±5825) primers. Reactions were performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and then elongation at 72 °C for 5 min. NF and C1 primers or TA-3 and C3R primers were used to amplify the *toxA* sequence from the plasmids, as applicable.
DNA from German ovine and porcine isolates and isolates from animals other than sheep (P. multocida) were semi-nested PCR amplified using primers: TA-1 and TA-2 for the primary reaction (865 bp product) and (b) TA-3 and TA-2 primers for the secondary reaction (691 bp product). Sequencing was performed using the TA-3 primer. (c) The N (receptor binding) domain and C1 (plasma membrane targeting) domains were amplified using NF and C1R primers (2157 bp product) and sequenced using the NF primer. (d) The C2 (function unknown) and C3 (G-protein activation) domains were amplified using TA-3 and C3R primers (1981 bp product) and sequenced using the TA-3 primer.

**Sequencing of toxA**

Semi-nested toxA PCR product was sequenced from clinical ovine P. multocida isolates and isolates from animals other than sheep (primary reaction amplification primers: TA-1+TA-2; secondary reaction amplification primers: TA-3+TA-2; sequencing primer: TA-3). In addition, semi-nested PCR and sequencing of toxA were performed using DNA from German ovine and porcine P. multocida strains, generously provided by Dr Jörg Rau.

Larger gene segments that had been cloned into plasmids were sequenced from ovine abattoir isolates (N+C1 domain amplification primers: TA-3+C3R; sequencing primer: TA-3). PCR products were gel excised using NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel) and sequenced by Beckman Coulter Genomics-Europe. Sequences were analysed in Sequencher 4.9 (Gene Codes, licensed to the Institute for Experimental Pathology at Keldur) and compared to the toxA GenBank sequence AF240778.

**16S rDNA and multilocus sequence typing**

The 16S rDNA gene was amplified and sequenced using forward 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 5'-AGAAAGGAGTTGATCCAGCC-3' primers (Beneditzi et al., 2010). The sequence was compared to the 16S GenBank sequence AY078999.1. P. multocida multilocus sequence typing (MLST) primers and amplifying conditions were based on those described by Subaaharan et al. (2010), with the addition of 18 bp tags on the primers, as described by Fujiiwara-Nagata et al. (2013). These tags allow bulk sequencing of different MLST genes, using only two primers (TagF: 5'-CAGGAACAGCTATGACC-3' and TagR: 5'-TGTTAAACACGGCCAGGCA-3'). Sequence type was determined based on the P. multocida RIRDC MLST database (http://pubmlst.org/pmultocida_rirdc/). Sequence data were graphed using ClustalX2 (Larkin et al., 2007) and MEGA6 (Tamura et al., 2013) using a minimum evolution tree with a bootstrap of 500 replicates; the number on each node indicates the percentage with which each topology branch was supported.

**Statistical analysis**

A burden test was used to test for genetic variability between groups of isolates using a two-tailed Mann–Whitney U test. The number of variable bases from the MLST sequencing data was counted for each isolate, and the sums were compared for clinical ovine isolates vs ovine abattoir isolates and heterogeneous isolates vs homogeneous isolates, as determined in Fig. 3.

**RESULTS**

**Capsule typing**

Capsule types were determined by screening for genes involved in capsule biosynthesis (Townsend et al., 2001). Abattoir bacteria were all capsule type D (Table 1, Figs 2 and 3, A1–A7; 19 isolates in total from 8 animals), whilst bacteria from sheep with respiratory symptoms had capsule type A, D or F (Table 1; Figs 2 and 3, C1–C10; 10 isolates in total from 10 animals). However, capsule type D was also the most prevalent in these bacteria.

**Toxigenicity of P. multocida**

Screening of Icelandic ovine P. multocida revealed that the toxA toxin gene was present in all 29 ovine isolates examined (Table 1), which was confirmed by sequencing of a 691 bp fragment of the toxin gene (Fig. 1). Twenty-eight P. multocida isolates from non-ovine sources in Iceland were also PCR screened for the toxin, with 13 bacterial isolates testing positive [bovine (1), porcine (2), mink (1), avian (5), feline (2) and canine (2)]. The PCR product from the Icelandic ovine and non-ovine bacteria, as well as two German toxigenic ovine and porcine strains, was sequenced. The 691 bp toxin sequence was identical in all ovine and non-ovine isolates, except for a silent base change (base 2358 C->T) in an Icelandic dog, a cat and a laying hen and one amino acid change (base 2693 T->A) in an Icelandic pig isolate.

In order to sequence a larger region, the toxin gene from abattoir strains was cloned in two segments (N and C1 domains or C2 and C3 domains, respectively; Fig. 1). Sequencing revealed that two isolates (A1a and A7a) had silent base changes in the N domain (base 275 T->C and...
Sequencing of 16S rRNA gene and MLST

Sequencing of the 16S rRNA gene revealed no variability in the Icelandic ovine \textit{P. multocida}. The majority of the ovine abattoir isolates was MLST 131 (Table 2), whilst one isolate had a novel allele combination (A7b) and one isolate had a novel \textit{adk} allele (A1a). None of the clinical ovine isolates had a known sequence type (C1–C10). The \textit{adk}, \textit{zwf} and \textit{mdh} genes all had previously described alleles, whilst none of the isolates had a known \textit{pgi} allele and there was some variability in the \textit{est}, \textit{pmi} and \textit{gdh} genes.

The sequence data for the seven MLST genes were compiled and graphed to evaluate the variability between isolates (Fig. 3). Ovine abattoir isolates A1–A7 and ovine clinical isolates C1–C4 were almost 100 % similar. C5–C10 were more variable, with 99.3 % similarity between C5–C6 and A2, 99.4 % similarity between C7 and A2 and 99.2 % similarity between C8–C9 and A2. C10 was the most distant, with 99.1 % similarity to A2. A burden test indicated that the ovine clinical isolates were significantly different from the abattoir isolates.

Table 1. Bacterial origin, toxigenicity and capsule type of Icelandic \textit{P. multocida}

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of isolates</th>
<th>toxA positive</th>
<th>toxA negative</th>
<th>Capsule type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep abattoir</td>
<td>19</td>
<td>19 (100 %)</td>
<td>0 (0 %)</td>
<td>D (100 %)</td>
</tr>
<tr>
<td>Sheep clinical respiratory symptoms</td>
<td>10</td>
<td>10 (100 %)</td>
<td>0 (0 %)</td>
<td>A (10 %) D (60 %) F (30 %)</td>
</tr>
<tr>
<td>Cattle</td>
<td>2</td>
<td>1 (50 %)</td>
<td>1 (50 %)</td>
<td>NA</td>
</tr>
<tr>
<td>Pig</td>
<td>4</td>
<td>2 (50 %)</td>
<td>2 (50 %)</td>
<td>NA</td>
</tr>
<tr>
<td>Mink</td>
<td>3</td>
<td>1 (33.3 %)</td>
<td>2 (66.6 %)</td>
<td>NA</td>
</tr>
<tr>
<td>Fowl</td>
<td>7</td>
<td>5 (71.4 %)</td>
<td>2 (28.6 %)</td>
<td>NA</td>
</tr>
<tr>
<td>Cat</td>
<td>6</td>
<td>2 (33.3 %)</td>
<td>4 (66.6 %)</td>
<td>NA</td>
</tr>
<tr>
<td>Dog</td>
<td>6</td>
<td>2 (33.3 %)</td>
<td>4 (66.6 %)</td>
<td>NA</td>
</tr>
</tbody>
</table>

\textbf{NA}, Not assayed.
the ovine abattoir isolates (two-tailed Mann–Whitney U test, \(P<0.05\)). Comparison of homogeneous bacteria (ovine abattoir isolates and ovine clinical isolates C1–C4) to heterogeneous bacteria (C5–C10) indicated that the difference between the two groups was highly significant (two-tailed Mann–Whitney U test, \(P<0.0001\)), despite the small number of samples.

**DISCUSSION**

The objective of this study was to examine the variability of Icelandic *P. multocida* isolates and search for virulence factors that could contribute to disease in Icelandic sheep. For this, we compared bacteria isolated from sheep with respiratory symptoms and apparently healthy slaughter animals.

Bacterial pulmonary infections caused by *Mycoplasma* and *Pasteurella* spp. are a significant problem in farm animals worldwide. Whilst the bacteria can be part of the normal upper respiratory tract flora, stress and immunosuppression can enable the bacteria to cause disease (Brogden et al., 1988; Dabo et al., 2007). An example of this is shipping fever pneumonia in cattle, where livestock shows respiratory symptoms after transport, which is often associated with a high mortality rate (Storz et al., 2000). Few suspected pasteurellosis cases are reported annually in Iceland, the majority of which are caused by *Mannheimia haemolytica* and some are treated by veterinarians without bacteriological confirmation. The difficulty in assessing the true incidence of *P. multocida* pneumonia in Icelandic sheep and collecting a sufficient number of isolates for analysis stems from the lack of systematic organ sampling and the small sheep population in Iceland. However, movement of animals between quarantine zones has been restricted since 1941, which enables comparison of bacterial isolates between regions, despite the isolates being collected years apart. As an example, isolates C5 and C6 were collected 7 years apart but have the same MLST profile and capsule type, which indicates that the observed heterogeneity between clinical ovine strains does not result from random mutations, but rather that the isolates are different strains which can be well conserved.

The bacterial isolates from abattoir sheep all had capsule type D and were remarkably well conserved, as assessed by comparing the MLST housekeeping gene sequences. Isolates from animals with respiratory symptoms, however, were more variable in terms of both capsule type and the housekeeping genes. Interestingly, more variability correlated with non-D capsule types within this group. At present,
Table 2. Multilocus sequence types

<table>
<thead>
<tr>
<th>Allele</th>
<th>Multilocus sequence type</th>
<th>Sample name</th>
</tr>
</thead>
<tbody>
<tr>
<td>adk est pmi zwf mdh gdh pgi</td>
<td>A1a</td>
<td>A1b, A2–A6, A7a</td>
</tr>
<tr>
<td>No matches found</td>
<td>C1–C3</td>
<td>Unknown sequence type</td>
</tr>
<tr>
<td>n=1/19 21 15 17 28 21 19 29</td>
<td>No matches found C7</td>
<td></td>
</tr>
<tr>
<td>n=2/10 4 4‡ 65 38 22 13§ 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=1/10 2 9 7¶ 8 7 3 9#</td>
<td>No matches found C9</td>
<td></td>
</tr>
<tr>
<td>n=1/10 2 7 5 6 4 5 7‡† No matches found C8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical respiratory disease</td>
<td>A7b</td>
<td>No matches found C10</td>
</tr>
<tr>
<td>n=3/10 21 15 17 28 21 19 29†</td>
<td>No matches found C4</td>
<td></td>
</tr>
<tr>
<td>n=1/10 21 15 17 8 21 19 29†</td>
<td>No matches found C5–C6</td>
<td></td>
</tr>
<tr>
<td>n=1/10 21 15 17 28 21 19 29†</td>
<td>No matches found C7</td>
<td></td>
</tr>
</tbody>
</table>

there are 40 publicly available complete or draft *P. multocida* genome sequences, predominantly from bovine, porcine and fowl strains. It will be interesting to compare Icelandic ovine *P. multocida* genomes and plasmids to available ovine and caprine data and to identify similarities and differences between the isolates, e.g. in terms of antibiotic resistance genes and insertion sequences (Boyce et al., 2012; Michael et al., 2012). Comparative genomic analysis of the heterogeneous and homogeneous ovine bacteria may, in addition, reveal possible virulence factors and better determine their phylogenetic relationships.

Increased genetic variability, determined by comparing the MLST gene sequences, correlates with disease propensity in the sheep, although isolates C1–4 and A7b appear to be exceptions to this rule. Isolate A7b has two mutations leading to an amino acid change in the toxA gene and two silent base changes in the zwf gene, compared to the other abattoir isolates. Increased variability of clinical isolates may be a result of a higher mutation rate owing to increased proliferation of the bacteria in diseased lungs, although the similarity of strains C5 and C6 (collected 7 years apart) and the different genes required for biosynthesis of the different capsules would suggest that the heterogeneous isolates represent different strains which can be conserved for extended periods of time. Whilst there are not enough data to see if a new, heterogeneous strain has appeared on farm A7, it will be interesting to sample lungs from this farm in the future.

toxA has been detected in *P. multocida* isolated from several animal species, including humans (Donnio et al., 1991). PMT, the toxin encoded by this gene, is a strong mitogen in a variety of cell types and activates a range of intracellular signalling pathways, indicating that the toxin could contribute to disease in various hosts (Essler et al., 1998; Orth & Aktories, 2010; Surguy et al., 2014). However, the primary focus of PMT research has been on porcine strains, and the toxin is generally thought to be absent or rare in other animal species (Harper et al., 2006; Jaglic et al., 2005; Verma et al., 2013; Weiser et al., 2003). Therefore, it was surprising to find toxA in all Icelandic ovine *P. multocida* isolates that were available for this study, ranging from apparently healthy slaughter animals to those that succumbed to pasteurellosis. Not all *P. multocida* from other animal species carried the toxA gene, which shows that it is not ubiquitously present in Iceland. The wide distribution and extensive conservation of the toxA gene in ovine *P. multocida* in this study could indicate that the toxin plays a role in the survival or spread of *P. multocida*. However, only a fraction of bacteria isolated from other animal species carried the toxA gene, which indicates that it is not necessary for survival of the bacterium. It is thus unclear whether mutations in the gene would have an impact on the fitness of the bacterium. Examination of the genes surrounding toxA in ovine strains would be interesting in order to see whether the bacteriophage containing toxA is situated in the unique 18 kbp region identified in a porcine strain (HN06) by whole genomic sequencing (Wilson & Ho, 2013) or whether the bacteriophage has integrated independently into the Icelandic *P. multocida* bacteria.

There have been significant improvements in *Pasteurella* vaccines in recent years, most notably the shift to growing the bacteria under iron-limiting conditions. Studies examining PMT regulatory elements indicate that limiting iron may also increase the quantity of PMT in bacterial cultures (Hoskins & Lax, 1996) and that the PMT toxoid induces potent immune responses that can protect both rodents and pigs in experimental infections (Jarvinen et al., 1998; Kim et al., 2012; Lee et al., 2012; Petersen et al., 1991; Seo et al., 2009; To et al., 2005). Based on the current work, capsule type should also be considered when *Pasteurella* vaccines are developed and administered. The findings suggest
that there are at least two groups of *P. multocida*, a genetically homogeneous group that may be found in the respiratory tract and a genetically heterogeneous group that could be the predominant cause of disease. Additional virulence factors may exist that distinguish between bacteria that can colonize the upper respiratory tract without causing disease and those that cause respiratory disease.

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