University of Veterinary Medicine Hannover

Clinic for Poultry

In vivo and in vitro investigations on *Bordetella avium* infection in turkeys

THESIS

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Für Mama und Papa
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Publications

Research articles:

Rebecca Knab, Henning Petersen, Hsuen-Ju Lin, Martin Meixner, Silke Rautenschlein, Arne Jung (submitted). *In vitro* characterization and genetic diversity of *Bordetella avium* field strains. Submitted for publication in *Avian Diseases*

Oral presentations at scientific meetings:
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Establishment of a *Bordetella avium* challenge model in turkeys. In H.M. Hafez (Ed.). Proceedings of the 11\textsuperscript{th} International Symposium on Turkey Diseases (pp. 120-123). Berlin, Germany

Henning Petersen, Rebecca Knab, Arne Jung, Silke Rautenschlein (2016).
Establishment of a *Bordetella avium* challenge model and development of a new generation vaccine for turkeys. *French-German Summer School for Promotion of Veterinary Science*, Bern, Switzerland

Establishment of a *Bordetella avium* challenge model and development of a new generation vaccine for turkeys.

Poster presentations at scientific meetings:
Pathogenesis of *Bordetella avium* monoinfection in turkey tracheal organ cultures and evaluation of different inoculation routes *in vivo*. *20\textsuperscript{th} World Veterinary Poultry Association Congress*, Edinburgh, United Kingdom
### List of abbreviations

**A.**  
Alcaligenes

AIR  
Air-liquid interface

AMPV  
Avian Metapneumovirus

ANOVA  
analysis of variance

**B.**  
Bordetella

**BA**  
*Bordetella avium*

**Baa**  
*Bordetella avium* autotransporter

**BhuR**  
Bordetella heme utilizing receptor

**bp**  
base pairs

**bvg**  
Bordetella virulence gene

**CALT**  
conjunctiva-associated lymphoid tissue

**cgMLST**  
core genome multilocus sequence typing

**CFU**  
colony forming units

**CSB**  
Columbia sheep blood

**°C**  
degree Celsius

**d**  
day

**DNA**  
deoxyribonucleic acid

**DNT**  
dermonecrotic toxin

**e.g.**  
exempli gratia

**ELISA**  
enzyme linked immunosorbent assay

**ESS**  
Earle’s Salt Solution

**et al.**  
et alii (and others)

**Exp.**  
experiment

**FBS**  
fetal bovine serum

**fha**  
filamentous hemagglutinin gene locus

**FHA**  
filamentous hemagglutinin

**Fig.**  
figure

**fim**  
fimbrial operon

**h**  
hour
Hag hemagglutinin
i.e. id est
lg immunoglobulin
IgA immunoglobulin A
IgG immunoglobulin G
IgM immunoglobulin M
KBE koloniebildende Einheiten
LPS lipopolysaccharide
MALDI-TOF MS matrix assisted laser desorption ionization-time of flight mass spectrometry
Mb mega base
µg microgram
µl microliter
MgSO₄ magnesium sulfate
m metre
min minute
ml milliliter
mm millimetre
NALT nasal-associated lymphoid tissue
no. number
OD optical density
Omp outer membrane protein
P/S penicillin/streptomycin
PBS phosphate-buffered saline
PCR polymerase chain reaction
pH potential hydrogenii
pi post inoculation/ infection
pv post vaccination
rpm rounds per minute
rRNA ribosomal ribonucleic acid
S Svedberg
TCT  tracheal cytotoxin
TOC  tracheal organ culture
TOK  Tracheal-Organ-Kultur
TPPPS  Taishan Pinus massoniana Pollen Polysaccharides
USA  United States of America
List of tables

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1. Summary

Rebecca Knab

In vivo and in vitro investigations on Bordetella avium infection in turkeys

*Bordetella avium* (BA) is a bacterial pathogen inducing respiratory disease in young turkeys. BA has a significant economic impact on turkey production worldwide and affects animal health and welfare. Knowledge about the pathogenesis of avian bordetellosis, control mechanisms against BA and virulence mechanisms is very limited. Preventive measures against the clinical disease induced by BA are restricted to general biosecurity measures and the application of vaccines, which have not yet proven to be reliable in protection of turkey flocks. Nevertheless, flock-specific autogenous vaccines are an important component of prevention strategies against BA-infection in European countries. Due to legal regulations, the pathogen has to be inactivated as a component of this type of vaccine. Consequently, the vaccine needs to be applied parenterally by individual injections to induce an effective immune response. The procedure of individual application has several disadvantages in commercial turkey husbandry: It is not only labor and cost intensive, but induces lesions in the skin and muscles at the application site of the vaccine and involves handling of the turkeys, which provokes stress reactions in the animals. On these grounds, the German Federal Ministry of Food and Agriculture funded a joint project with the goal of the “development of innovative flock-specific vaccines for simplified application” (InnoVak4DART), in which the two studies, which are included in the present thesis, were embedded.

The aims of the first study (chapter 4), were to enhance the understanding of the pathogenesis of BA-infection and to establish a reliable *in vivo* model to test efficacy of BA-vaccines against BA-challenge.
The objectives of the first experiment in the study were to investigate the effects of different dosages and inoculation routes on the pathogenesis of BA-infection and on the immune response to the pathogen. BA-pathogenesis was experimentally investigated in naïve turkeys, which were inoculated at the age of 28 days. The turkeys were either inoculated oculonasally with 10⁵, 10⁷ or 10⁹ colony forming units (CFU) of BA per bird or exposed to BA by aerosol with 10⁵–10⁸ CFU/m³. The four differently inoculated groups and a BA-free control group were compared regarding the clinical signs, BA-colonisation pattern in choanae, tracheae and lungs and the humoral systemic and local BA-specific immune responses. In comparison to the oculonasally inoculated groups, aerosol inoculation led to higher prevalence and to delayed clearance of BA from the respiratory tract of the turkeys. Circulating BA-specific IgG was detected from five days post inoculation (pi) onwards and IgA in lacrimal fluid from seven days pi, regardless of the applied inoculation route and dose. In all groups, antibodies remained at a high level with a slightly downward tendency until the end of the experiment 21 days pi.

The objective of the second experiment in the first study was to ascertain the reliability of the model for the evaluation of the efficacy of a vaccine against BA. 21-day-old turkeys were vaccinated with a formalin-inactivated BA-vaccine intramuscularly and challenged 21 days post vaccination (pv) with 10⁷ CFU per bird oculonasally. BA-specific IgG antibodies were detected in serum as well as in lacrimal fluid at 14 days pv. No BA-specific IgA-antibodies were measured in lacrimal fluids. Vaccination induced partial protection against the pathogen. The tracheae of vaccinated animals were colonised with a significantly lower number of BA compared to the non-vaccinated animals (P ≤ 0.05). Consequently, the established model is suitable for vaccine efficacy testing even in the absence of clinical signs or pathological alterations.

The aim of the second study of this project (chapter 5) was to characterise BA field strains regarding their virulence-associated properties and their putative virulence mechanisms. The first objective was the establishment of two in vitro assays in
tracheal organ cultures (TOC) to characterise the strains regarding their ability to adhere to the tracheal epithelium and to induce ciliostasis. The second objective was to compare BA field strains regarding these virulence-associated properties in the established \textit{in vitro} models. The third and fourth objectives of this study were to investigate genetic diversity of the included BA field strains and to identify possible genetic factors for reduced virulence-associated properties.

In this study, we included 17 BA-strains, which had been isolated from several poultry flocks. Following inoculation of TOCs with \(10^5\) and \(10^7\) CFU/ml, three strains displayed a reduced ability to adhere to the tracheal epithelium compared to the other strains. Additionally, the ciliostasis assay demonstrated a significantly reduced and delayed ciliostatic effect after inoculation with these three strains for both inoculation doses compared to the other strains \((P \leq 0.05)\). The results demonstrate that BA field strains differ in their ability to adhere and to induce ciliostasis. The characteristics of adherence and ciliostasis were reproducible in experiments with two different inoculation dosages and the ability to adhere to the tracheal epithelium and to induce ciliostasis covaried.

Core genome multilocus sequence typing (cgMLST) was performed to investigate the genetic diversity of the strains and to find out whether the strains with reduced virulence-associated properties are closely related to each other regarding their virulence-associated genes. Next generation sequencing data of 45 BA field strains, which had been gathered by our cooperative partners, and two published BA-genomes were used to develop a cgMLST scheme based on 2,667 targets of the reference genome, which was equivalent to 77.3\% of the complete genome. cgMLST analysis of the 17 investigated field strains demonstrated a genetic diversity in the BA field strains. A minimum spanning tree including 119 virulence-associated genes showed that the number of allele differences in these genes varied between one and 71. Most of the investigated strains formed a group with only one to 15 allelic differences in virulence-associated genes. The three strains with reduced virulence-associated properties were part of this group. A correlation between the
sequence type data and the virulence-associated properties could not be detected with the available data. However, the cgMLST analysis together with amino acid sequence alignment suggest a potential candidate contributing to reduced virulence as strains with reduced virulence-associated properties had mutations in the putative Filamentous Hemagglutinin (FHA) protein. Mutations in that protein may modify the ability of the bacteria to adhere to the tracheal epithelium, to colonise the respiratory tract of turkeys and subsequently to induce pathological alterations.

Overall, this work provides new insights into the pathogenesis of BA-infection in turkeys. It suggests that humoral immunity may play an important role in protection against the infection as antibody-positive birds showed reduced BA-colonisation of the respiratory tract. The knowledge about humoral protection against colonisation of the respiratory epithelia can be of relevance for the development of vaccine strategies. Additionally, we established a challenge model, which can be used for efficacy testing of innovative vaccines, which will be developed in the future. The challenge model can be modified for application of vaccines via different application routes, such as “eyedrop” (ocularnasal application) or aerosolisation of vaccines. In addition, we could describe variations in virulence-associated properties and virulence-associated genes of BA field strains in vitro. These variations should be characterised in more detail in future studies, including their effect on variations in protein structure and protein function and their relevance for the virulence phenotype of the strains in vivo. This knowledge could possibly provide further opportunities for BA-vaccine development.
2. Zusammenfassung

Rebecca Knab

*In vivo* und *in vitro* Untersuchungen zur *Bordetella avium* Infektion der Pute


Die Ziele der ersten Studie (Kapitel 4) waren, das Verständnis der Pathogenese der BA-Infektion zu verbessern und ein verlässliches *in vivo*-Modell zur

Schutz gegen den Erreger, was durch die quantitative Analyse der BA-Besiedlung der Trachea nach der Belastungsinfektion nachgewiesen werden konnte. Die Besiedlung der Trachea geimpfter Tiere war signifikant geringer als die der nicht geimpften Tiere \( (P \leq 0.05) \). Folglich ermöglicht dieses Infektionsmodell, die Wirksamkeit eines Impfstoffes auch ohne die Induktion klinischer Symptome oder pathologischer Veränderungen zu beurteilen.


In diese Studie wurden 17 BA-Stämme aufgenommen, die aus verschiedenen Geflügelbeständen isoliert worden waren. Nach einer Inokulation der TOK mit \( 10^5 \) und \( 10^7 \) KBE/ml zeigten drei Stämme eine verringerte Adhäsionsfähigkeit. Zusätzlich zeigte der Ziliostase Assay, dass die drei Stämme unter Verwendung beider Inokulationsdosen auch einen signifikant verminderten oder verspäteten ziliostatischen Effekt im Vergleich zu den anderen Stämmen besaßen \( (P \leq 0.05) \). Die Eigenschaften von Adhäsion und Ziliostase waren reproduzierbar in Versuchen mit zwei verschiedenen Inokulationsdosen und die Fähigkeit zu adhärenieren und die Ziliaktivität zu hemmen kovarierten.

Zur Untersuchung der genetischen Diversität der Stämme und um der Frage nachzugehen, ob die Stämme mit reduzierten virulenz-assoziierten Eigenschaften eine enge Verwandtschaft ihrer virulenz-assoziierten Gene aufweisen, wurde ein

Somit bietet diese Arbeit neue Einblicke in die Pathogenese der BA-Infektion der Pute. Sie weist auf eine möglicherweise wichtige Rolle der humoralen Immunantwort im Schutz gegen die Infektion hin, da antikörper-positive Vögel eine reduzierte BA-Besiedlung des Respirationstraktes zeigten. Das Wissen über den humoralen Schutz gegen die Besiedlung der respiratorischen Epithelien kann für die Entwicklung neuer Impfstrategien von Bedeutung sein. Wir konnten außerdem ein Infektionsmodell etablieren, das für die Wirksamkeitsprüfung innovativer Impfstoffe genutzt werden kann, die in Zukunft entwickelt werden. Das Belastungsmodell kann für verschiedene
3. Introduction

3.1. **Bordetella avium (BA) infection of turkeys**

3.1.1. Aetiology

3.1.1.1. Genus *Bordetella*

The genus *Bordetella* (B.) belongs to the bacterial family of *Alcaligenaceae* within the class of Betaproteobacteria. It includes the species *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium* (BA), *B. hinzii*, *B. holmesii*, *B. petrii*, *B. ansorpii* and *B. trematum*. Results of a genome-wide sequence comparison and gene content analysis between the *Bordetella* species suggest an evolution of the *Bordetella* genus into three distinct clades (Linz et al., 2016). The first clade includes the so-called classical *Bordetellae* *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*. *B. bronchiseptica* is a respiratory pathogen with a broad spectrum of mammalian hosts (Goodnow, 1980; Mattoo & Cherry, 2005). *B. parapertussis* is known to induce respiratory diseases in human and sheep (Hester et al., 2015) and *B. pertussis* has specific host tropism for humans. The clinical disease induced by the latter is known as whooping cough and can have severe manifestations especially in non-vaccinated children and elder people. The second cluster consists of *B. holmesii*, *B. trematum*, *B. hinzii* and BA. *B. holmesii* induces a pertussis-like respiratory disease in humans and shows high genetic similarity to *B. pertussis* (Harvill et al., 2014; Njamkepo et al., 2011). In contrast to the majority of the *Bordetellae*, *B. trematum* has no tissue tropism for the respiratory tract, but has been isolated from local inflammatory processes in wounds and ears of human patients (Vandamme et al., 1996). *B. hinzii* has been isolated from poultry (Vandamme et al., 1995) and rodents (Jiyipong et al., 2013) with respiratory clinical signs and from immunocompromised humans affected by respiratory and other symptoms (Cookson et al., 1994; Gadea et al., 2000). BA is highly specialised in avian hosts. Isolated from turkeys, which were affected by a respiratory disease, named turkey rhinotracheitis, the pathogen was first designated as *B.-bronchiseptica*-related bacterium (Filion et al., 1967) or *Alcaligenes faecalis*. 
(Saif et al., 1980) until it was realised that the bacterial cause of the disease represented a new species distinct from \textit{A. faecalis} and the previously known 
\textit{Bordetellae} (Hinz et al., 1983). It was demonstrated that the pathogen belongs to a separate bacterial species which was classified as \textit{Bordetella avium} sp. nov. (Kersters et al., 1984). Further molecular characterisation confirmed its unique taxonomic position within the genus \textit{Bordetella} (Jackwood et al., 1986; Moore et al., 1987; Spears et al., 2003). The opportunistic species \textit{B. petrii} and \textit{B. ansorpii} form the third clade of the genus \textit{Bordetella}. Knowledge about these two species is very limited. \textit{B. ansorpii} has been found in wound infections (Ko et al., 2005) and \textit{B. petrii} has been isolated from the environment as well as from diverse localised infections in humans (Fry et al., 2005; Gross et al., 2008).

\textbf{3.1.1.2. Genome}

Until today, two BA-genomes are published (Moreno et al., 2015; Sebaihia et al., 2006). In comparison to other \textit{Bordetella} species, the BA-genome is relatively short with a length of approximately 3.73 Mb. (Beach et al., 2012) and has an overall similarity of 97\% regarding the nucleotides and 75\% regarding the proteins, with \textit{B. pertussis}, \textit{B. parapertussis} and \textit{B. bronchiseptica} (Beach et al., 2012). It has been demonstrated that through co-evolution of sequence and gene content from a common ancestor to species-specific pathogens, many genes were lost. This is reflected by a large variation in genome size and differences in virulence-associated genes between the \textit{Bordetella} species clades (Linz et al., 2016). Beside loss of multiple genes, evolution of BA includes an acquisition of several BA-specific genes which could possibly be of relevance for virulence-associated properties of the species (Sebaihia et al., 2006).

\textbf{3.1.1.3. Morphology}

Microscopically, BA is a gram-negative, motile rod. It grows under strictly aerobic conditions (Jackwood et al., 1985; Kersters et al., 1984). Three different colony types have been described. The most frequent manifestation are translucent colonies with smooth edges and a diameter of 0.2 -1 mm after 24 h and 1-2 mm after 48 h of incubation (Kersters et al., 1984). Some strains are characterised by larger colonies
of that type (Hinz et al., 1983). A rough colony type with a dry appearing surface and irregular edges has also been described and some strains dissociate in different types. Some authors hypothesise that rough colonies are non-pathogenic (Jackwood et al., 1991).

### 3.1.1.4. Virulence factors and mechanisms

Broad similarities in pathogenesis of the clinically relevant *Bordetellae* support the hypothesis that bacterial factors for infection and disease induction may be similar between these different species. Most *Bordetella* species have a strong tropism for the ciliated respiratory tissue of their respective host. Adherence to the ciliated epithelium and subsequent ciliostatic effects compromise the clearance activity of the respiratory tract (Anderton et al., 2004). However, there are some known and notable differences in virulence-associated mechanisms between the mammalian adapted *Bordetella* species and BA. An overview on virulence factors of BA in comparison to *B. pertussis* and *B. bronchiseptica* is summarised in Table 1.
Table 1. Virulence-associated key factors in genomes of *Bordetellae*.
Adapted from (Linz et al. 2016)

<table>
<thead>
<tr>
<th>Virulence-associated key factor</th>
<th>B. pertussis</th>
<th>B. bronchiseptica</th>
<th>B. avium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bordetella virulence gene locus (BvgAS)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Filamentous Haemagglutinin (FHA)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BA haemagglutination (HagA, HagB)</td>
<td>-</td>
<td>-</td>
<td>+(^1)</td>
</tr>
<tr>
<td>Dermonecrotic Toxin (DNT)</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Fimbrial operon (fimA-D)</td>
<td>+(^2)</td>
<td>+(^2)</td>
<td>+(^3)</td>
</tr>
<tr>
<td>BA fimbrial operon</td>
<td>-</td>
<td>-</td>
<td>+(^3)</td>
</tr>
<tr>
<td>Type 1 secretion system-Adenylate Cyclase Toxin</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Type 2 secretion system</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Type 3 secretion system</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Type 4 secretion system-Pertussis Toxin</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Type 5 secretion system-Pertactin</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Type 6 secretion system</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>O-antigen A</td>
<td>-</td>
<td>+/(^{-})</td>
<td>-</td>
</tr>
<tr>
<td>O-antigen B</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Capsule A</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Capsule B</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose synthesis</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flagella</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligin receptor</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Heme receptor</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Enterobactin receptor</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^1\) (Temple et al., 2010); \(^2\) (Parkhill et al., 2003); \(^3\) (Sebaihia et al., 2006)
3.1.1.4.1. *Bordetella* virulence gene locus (bvg)

It has been shown for the mammalian host-adapted *Bordetellae* that the majority of the virulence factors is regulated by a nearly identical control system, the *Bordetella* virulence gene locus (bvg) (Arico et al., 1991; Scarlato et al., 1991b) which is responsive to environmental conditions. The bvg locus consists of two genes, bvgA and bvgS. Their gene products BvgA and BvgS form a two component signal transduction system which uses a multistep transfer of phosphoryl groups to regulate gene expression pathways (Uhl & Miller, 1994). Under laboratory conditions, bvgAS is active when the bacteria are grown at 37°C in the absence of MgSO₄ and nicotinic acid. Under these conditions, virulence-associated genes are expressed (Gross & Rappuoli, 1988; McPhear et al., 1983; Roy et al., 1990; Scarlato et al., 1990). Environmental conditions characterised by low temperatures, presence of nicotinic acid or MgSO₄ cause the bvgAS to be inactive and the expression of virulence-associated factors is inhibited. A third intermediate phenotypic phase has also been described, which is characterised by expression of adhesins and immunogenic surface structures and no expression of toxins (Scarlato et al., 1991a). The sensory transduction system allows the *Bordetellae* to change their phenotype depending on environmental influences. Phenotypic phase variation represents a mechanism of adaptation to conditions inside the respiratory tract of a host on the one side, and conditions outside a suitable host on the other side (Cotter & Jones, 2003). The bvg activated phase is necessary for the establishment of a respiratory infection and the bvg inactivated phase allows the pathogen to survive under conditions of nutrient deprivation (Cotter & Miller, 1994; Merkel et al., 1998). The intermediate phase is characterised by the presence of antigens that are maximally or exclusively expressed in this phase and which are suspected to be necessary for aerosol transmission of the pathogen (Stockbauer et al., 2001).

Phase variation was also demonstrated for BA (Gentry-Weeks et al., 1991). However, first attempts to detect the bvgAS locus in BA using Southern DNA hybridisation of BA chromosomal DNA with DNA probes specific for bvgAS of *B. pertussis* failed partly. Digested chromosomal DNA from six BA strains did not
hybridise with an internal 509-bp DNA fragment of the *B. pertussis* bvgA gene, while hybridisation was successfull between this fragment and chromosomal DNA of other *B. pertussis* strains and one *B. bronchiseptica* strain. In contrast, digested chromosomal DNA of the six BA strains hybridised to the *B. pertussis* bvgS-specific probe (Gentry-Weeks et al., 1991). The authors anticipated the possibility that the partial failure of BA-bvgAS detection might not be a proof for the absence of the bvgA, but rather could be explained by the BA-bvgA gene lacking significant DNA homology to *B. pertussis* bvgA gene. These speculations were confirmed by later studies. BA possesses a bvgAS locus and the arrangement of the bvgA-bvgS gene pair is identical in BA, *B. pertussis* and *B. bronchiseptica* (Spears et al., 2003), but its arrangement in relation to other genes is different in BA in comparison to other *Bordetella* species. In contrast to *B. bronchiseptica* and *B. pertussis*, bvgAS of BA is not directly linked to the gene cluster of the fimbrial operon (fimABCD) and the filamentous hemagglutinin loci (fhaBC) (Sebaihia et al., 2006; Spears et al., 2003). Based on genome sequence comparison and comparison of predicted proteins, it has been shown that the predicted BvgS proteins share only about 45% identity between the group of the so-called ‘classical’ *Bordetella* species, which includes *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*, and the group of genetically more distantly related *Bordetellae*, which includes BA, *B. hinzii*, *B. trematum* and *B. holmesii* (Linz et al., 2016). Regarding the functionality of bvgAS, it was confirmed that despite the differences in DNA sequence, the predicted BvgA protein retained its functional structure for the phosphorelay mechanism, whereas the predicted BA-BvgS protein retained its phosphorelay sites only partly, because of a frameshift which predicts an earlier termination of the protein compared to other sequenced *Bordetellae* (Sebaihia et al., 2006). The shorter BvgS protein may lack the third phosphorelay residue that is essential for phosphorylation of BvgA in the other *Bordetellae*. As a functional BvgS was reported to be essential for virulence (Spears et al., 2003), it is suggested that the shorter predicted BvgS protein is functional, but the mechanism may differ from that of the other *Bordetellae* (Sebaihia et al., 2006).
3.1.1.4.2. Adhesins, haemagglutinins and fimbriae

BA and most of the other Bordetella species share the ability for adhesion to the tracheal epithelium. Adhesins, which are surface structures of the bacteria, are relevant for that kind of host-pathogen interaction and for colonisation of the host (Edwards et al., 2005; van den Berg et al., 1999). The major adhesin and hemagglutinin in the mammalian-adapted Bordetellae is Filamentous Haemagglutinin (FHA) (Kimura et al., 1990; Relman et al., 1989). It has been shown that FHA expression in B. bronchiseptica requires the presence of the genes fhaB and fhaC, as the product FhaB is a preprotein of FHA (Jacob-Dubuisson et al., 2000; Willems et al., 1994). FhaC is a secretory protein, necessary for the export of FhaB to the cell surface (Jacob-Dubuisson et al., 2000). In BA, orthologues of fhaB and fhaC are present (Sebaihia et al., 2006; Spears et al., 2003) and comparable to B. bronchiseptica. FhaB-negative mutants of BA show a significantly reduced ability to colonise the respiratory tract of one-day-old turkey poults (Spears et al., 2003). However, fhaB of BA shows only a low percentage of sequence similarity to the fhaB found in B. bronchiseptica (Linz et al., 2016; Sebaihia et al., 2006). Furthermore, the fhaB of BA is not directly linked to the bvgAS locus as it is the case in B. bronchiseptica and B. pertussis (Spears et al., 2003; Stibitz et al., 1988), indicating differences in genetic organisation. Consequently, it is not clear if fhaB is transcriptionally regulated by bvgAS as it is the case in B. pertussis and B. bronchiseptica. But the fact that mutations either in bvgS or fhaB both lead to attenuation of BA suggests such a relation (Spears et al., 2003).

It has been shown for B. bronchiseptica that virulent strains are able to agglutinate guinea pig erythrocytes (Nagano et al., 1988) and that FHA is responsible for hemagglutination in that species (Cotter et al., 1998; Locht et al., 1993). The correlation between hemagglutination and virulence has also been demonstrated for BA (Gentry-Weeks et al., 1988; Jackwood et al., 1985; Temple et al., 1998), but FHA seems not to be responsible for hemagglutination. FHA-negative mutants of BA retain the ability for haemagglutination and hemagglutination-negative phenotypes have no mutation in the fhaB gene (Spears et al., 2003). These findings indicate that
BA possesses a hemagglutination mechanism that is different from the mammalian-adapted *Bordetellae* and is necessary for the pathogen-host interaction.

Two additional, BA-specific genes involved in hemagglutination activity have been found and named hagA and hagB (Temple et al., 2010). They have no orthologues in the genomes of other *Bordetellae*. The product HagB was demonstrated to have a similar function in BA as does FhaB in the other Bordetella species. It is directly involved in hemagglutination and binding of the pathogen to the tracheal epithelium (Temple et al., 2010). For HagA, such a direct role in virulence-associated properties could not be proven. Protein analysis demonstrated structural similarity between HagA of BA and FhaC of *B. pertussis* (Clantin et al., 2007; Spears et al., 2003; Temple et al., 2010). As HagA shows structural similarities to FhaC and HagB shows functional similarities to FhaB, it is speculated that the HagA-HagB system has a similar function for hemagglutination and tracheal adherence as the FhaB/FhaC system has in the other *Bordetella* species.

Beside haemagglutinins and adhesins, fimbriae play an important role in tracheal colonisation (Mattoo et al., 2000). The genome of the mammalian-adapted *Bordetella* species contains a single operon (fimA-D) that codes the synthesis of fimbriae in these species (Kania et al., 2000; Parkhill et al., 2003; Willems et al., 1992). The expression of this operon is regulated by bvgAS and consequently depends on temperatures of approximately 37°C (Cummings et al., 2006). BA possesses a gene cluster that is homologous to the fimA-D operon of the mammalian *Bordetellae*. This operon is functional and virulence-associated in BA (Spears et al., 2003). Similar to the mammalian-adapted *Bordetellae*, the expression of this operon is also significantly higher at 37°C and probably regulated by the bvg locus (Loker et al., 2011; Sebaihia et al., 2006). Additionally, the BA-genome contains a second species-specific fimbrial operon and a higher number of fimbrial subunit genes than the other *Bordetella* species (Sebaihia et al., 2006). In contrast to the other *Bordetella* species, fimbria-like structures can be found at low temperatures of 22°C on the surface of BA. It was suggested that these fimbriae are a product of the second, BA-specific fimbrial operon (Loker et al., 2011). It is suggested that the first type fimbriae are
necessary for pathogenesis while the second type fimbriae might be relevant for survival in the environment. The BA-specific fimbriae of the second fimbrial operon may play only a minor role in the adherence to the host epithelium (Loker et al., 2011).

### 3.1.1.4.3. Toxins

Localised acute damage in the tracheal epithelium of BA-inoculated turkeys has been attributed to toxins produced by the pathogen (Gray et al., 1981; Gray et al., 1983b; Marshall et al., 1984). Several toxins found in *B. pertussis*, such as pertussis toxin and extracytoplasmic adenylate cyclase have no homologues in BA (Gentry-Weeks et al., 1988; Linz et al., 2016; Rimler & Rhoades, 1986; Sebaihia et al., 2006). On the contrary, the heat-labile Dermonecrotic toxin (DNT) known from *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* is also produced by BA (Gentry-Weeks et al., 1988). Notably, while the DNTs of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* show a high degree of similarity to each other, the predicted protein sequence of the toxin shows only 41% identity between *B. bronchiseptica* and BA and the DNT gene is located differently within the genome (Linz et al., 2016; Sebaihia et al., 2006). However, DNT produced by BA has pronounced toxic effects that are comparable to those of the DNT of the other *Bordetella*. DNT of all *Bordetella* species induces localised necrotic lesions following intradermal injection in rodents, and for BA-DNT, this is also the case in young chickens and turkey poults. Additionally, BA-DNT is lethal for these animals after intravenous injection (Gentry-Weeks et al., 1988; Iida & Okonogi, 1971; Kume et al., 1986; Parton, 1985). DNT is suspected of playing a role in the pathogenesis of BA-infection, as it was demonstrated that DNT-negative BA-mutants were avirulent in turkeys and had a reduced ability to colonise the tracheal epithelium *in vitro* (Temple et al., 1998).

A further toxin produced by *B. pertussis*, *B. bronchiseptica*, *B. parapertussis* and BA is the tracheal cytotoxin (TCT). It is involved in lesion development in the ciliated epithelium of the trachea following infection by causing destruction of ciliated cells via induction of nitric oxide biosynthesis (Goldman & Herwaldt, 1985; Heiss et al., 1993). While the TCTs of different *Bordetella* are chemically very similar, the amounts of
released TCT varies massively between the species with BA releasing only low quantities of the toxin compared to B. pertussis (Gentry-Weeks et al., 1988). A recent study in tracheal explant cultures also indicated that TCT is produced only in low concentrations and may have no significant impact on pathogenesis of BA-infection (Miyamoto et al., 2011).

3.1.1.4.4. Flagellae
It is known from *B. bronchiseptica* that flagellae and flagellin play a role in motility and host cell attachment as well as the immune reaction of the host (Lopez-Boado et al., 2005; Savelkoul et al., 1996). In the genus *Bordetella*, genes for flagella synthesis are located in a locus together with genes for chemotaxis (Linz et al., 2016). Flagella synthesis is negatively controlled by the bgvAS system, which was demonstrated for *B. bronchiseptica* (Akerley & Miller, 1993). Some authors state a minor contribution of flagellae to virulence of BA (Moore et al., 1994), but studies that focus on correlation of flagella synthesis and virulence are lacking.

3.1.1.4.5. Secretion systems, autotransporters and heme receptor
Protein secretion systems of Gram-negative bacteria are thought to contribute to pathogen virulence. The mammalian adapted *Bordetella* species possess secretion systems I, III, IV, V and VI. While the type III secretion system and type IV secretion system are important for virulence of *B. bronchiseptica* and *B. pertussis*, respectively (Cheung et al., 2004; Rambow-Larsen & Weiss, 2004; Yuk et al., 2000), BA is lacking these secretion systems (Linz et al., 2016). In contrast to the mammalian adapted *Bordetellae*, BA possesses a gene cluster that potentially encodes a type II secretion system. The function of this system is unknown in BA (Sebaihia et al., 2006).

Autotransporters are also relevant for pathogen-host interaction (Henderson & Nataro, 2001). For BA, seven intact autotransporter genes are described (Sebaihia et al., 2006). Only one of the autotransporter proteins (Baa1) has been investigated in
more detail. It was shown that it acts as a factor for attachment to the tracheal epithelium (Stockwell et al., 2011).

A further virulence-associated property in Gram-negative bacteria is the ability to acquire iron from the environment. An outer membrane heme receptor (bhuR) of BA was shown to be of relevance for the colonisation of the respiratory tract of turkeys. In a competitive challenge model, a mutant strain without expression of the receptor and a wild type strain were oculonasally applied to two-day-old turkey poults at the same time and with the same dosage. Two weeks post infection, the BA colonisation of the tracheae of the turkey poults was analysed quantitatively. As only 10% of the recovered BA cells were the bhuR-negative mutant and 90% were the wild-type strain, the authors concluded that the mutant strain without expression of the receptor was inferior to the wild type regarding colonisation of the respiratory tract of turkey poults. However, the induction of clinical signs was not affected by the lack of bhuR (Murphy et al., 2002).

3.1.1.4.6. Lipopolysaccharides and capsules

Lipopolysaccharides (LPS) also play a role during the infection with *Bordetella* species. It was demonstrated that LPS mutant strains of BA have decreased capability to colonise tracheae and to persist in naïve turkey serum *in vitro* (Spears et al., 2000). The LPS of BA show some structural similarity to the LPS of *B. bronchiseptica*. However, in contrast to *B. bronchiseptica*, the transport system for the O-antigen, which is an important part of the LPS, could not be identified in BA. It is suggested that the biosynthesis pathway for the expression of the O-antigen differs between the two species (Sebaihia et al., 2006)

Capsules are often considered as virulence factors as they contribute to the resistance of a pathogen against the defence mechanisms of the host. BA and the mammalian-adapted *Bordetellae* probably produce different types of capsular polysaccharides since BA possesses a capsule synthesis locus that is genetically different to the corresponding locus in the mammalian-adapted *Bordetella* species
Moreover, the presence of a bacterial cellulose synthase (bcs) operon that is similar to the one in *Pseudomonas* species has been described for the BA-genome (McLaughlin et al., 2017; Sebaihia et al., 2006). Additionally, it was shown that BA expresses cellulose in air-liquid interfaces (McLaughlin et al., 2017). The functional role of the cellulose synthesis has not been investigated in detail, but it is known, that cellulose production is correlated to the ability to form biofilms. This may be an additional virulence factor of BA.

**3.1.2. Epidemiology**

**3.1.2.1. Occurrence and distribution**

The first report on a *Bordetella*-related disease in a Canadian turkey flock was given by Filion et al. (Filion et al., 1967), followed by reports from Germany (Hinz et al., 1978). Further reports with very similar respiratory symptoms in turkeys were recorded, but the aetiology of these diseases could never be clarified. In some cases, an adenovirus was supposed to be the causative agent (Blalock et al., 1975) while in other cases, *Alcaligenes faecalis* came under suspicion (Panigrahy et al., 1981; Saif et al., 1980). Since it had been shown that BA is a possible responsible agent of respiratory disease (Kersters et al., 1984), the pathogen was detected in many countries. Even though a detailed analysis has not been performed so far, it is stated that avian Bordetellosis has an economic impact on turkey production worldwide (Clark et al., 2009; Jackwood & Saif, 2013).

**3.1.2.2. Transmission**

BA can be transmitted through direct contact between birds and due to its high contagiosity, even an indirect transmission through exposure to infected material is possible (Simmons & Gray, 1979). The survival time in the environment can be prolonged by low temperatures, dry conditions and neutral pH. Contaminated litter can remain infective for one to six months (Cimiotti et al., 1982). High tenacity plays a role in reinfections of consecutive flocks in one turkey house. Vertical transmission of the pathogen has not been reported.
3.1.2.3. Host spectrum

BA can infect a broad range of wild and domestic birds (Raffel et al., 2002; Stenzel et al., 2017). Clinical cases have been reported in wild turkeys (Hopkins et al., 1990), cockatiel chicks (Fitzgerald et al., 2001; Grespan et al., 2012) and quail (Odugbo et al., 2006). Additionally, BA plays a role as an opportunistic pathogen in chickens (El-Sukhon et al., 2002; Jackwood et al., 2009; Spilker et al., 2008) but clinical cases are rarely reported. Of all the investigated avian species, commercially raised turkeys show the highest susceptibility. Mainly young turkeys at the age of two to six weeks are affected by avian bordetellosis (Hinz et al., 1978; Kersters et al., 1984; Saif et al., 1980). When BA acts in coinfections with other pathogens, such as Avian Metapneumovirus (AMPV), susceptibility can be extended to older birds (Jirjis et al., 2004).

3.1.3 Pathobiology in turkeys

3.1.3.1. Pathogenesis

Avian bordetellosis is a disease of the upper respiratory tract of turkeys. The pathogen adheres highly specific to the ciliated epithelium in the respiratory tract of the host (Gray et al., 1981; Miyamoto et al., 2011; Soane et al., 2000; Temple et al., 1998) and induces localised lesions in the epithelium (Arp & Cheville, 1984; Temple et al., 1998). The ability to adhere to the tracheal mucosa is a requirement for the pathogenic effect of BA and it has been shown that there is a strong correlation between the ability to adhere to the tracheal mucosa and the ability to colonise turkeys (Temple et al., 1998). Ciliostasis, apoptosis and extrusion of ciliated cells from the epithelia are microscopically visible effects of the pathogen-host interaction (Miyamoto et al., 2011). Apoptosis in this case is discussed to be a defence mechanism against the pathogen as it results in removal of the bacteria from the mucosal surface, while the epithelial barrier is not reduced in its integrity (Miyamoto et al., 2011). On the other hand, the epithelial damages, which are induced by BA,
result in a reduction of the clearance function of the respiratory tract. This effect paves the way for secondary infections.

Until now, comparative studies investigating differences in pathogenesis and virulence between BA field strains are rare. One study including 128 *Alcaligenes faecalis* isolates describes two subtypes of that bacterial species of which the first subtype displayed pathogenic effects in turkeys, while the second subtype was apathogenic (Jackwood et al., 1985). *A. faecalis* had originally been considered to be identical with BA, but in more recent studies, it is speculated that these subtype II isolates might have been classified incorrectly and more likely were representatives of the *B. hinzii* species (Register et al., 2003). A comparative *in vitro* study including ten BA strains, which were isolated from cockatiels and turkeys, did not find differences in the investigated virulence-associated properties *in vitro*, namely attachment to tracheal rings obtained from one euthanised cockatiel chicks and cytotoxic effects on monkey kidney cells (Grespan et al., 2012).

### 3.1.3.2. Clinical signs

The incubation period may vary after different inoculation routes. While natural infections under field conditions result in an incubation period of seven to ten days, the period can be shortened to four to six days if the animals are experimentally inoculated via the intranasal route (Arp & Cheville). Reports about clinical signs include ocular-nasal discharge, coughing, sneezing (Arp & Fagerland, 1987; Saif et al., 1981), beak breathing, submandibular edema, tracheal collapse and stunted growth (Arp & Cheville, 1984). In general, flocks affected by avian bordetellosis display high morbidity and low mortality (Hinz et al., 1978; Saif et al., 1980), but mortality can increase after secondary infection with *Escherichia coli* (Saif et al., 1980) or AMPV (Cook et al., 1991; Jirjis et al., 2004). Usually, clinical signs decline after two to four weeks (Boycott et al., 1984; Gray et al., 1983a; Saif et al., 1980).
3.1.3.3. Gross pathology

Gross pathology is dominated by lesions in the upper respiratory tract. Sinusitis with nasal exudates of variable quality may be observed, as well as tracheitis with exudate, hyperemia, thickened tracheal walls and further tracheal lesions, such as softening, distortion and collapse of tracheal rings (Arp & Cheville, 1984; Arp & Fagerland, 1987).

3.1.3.4. Histopathology

In histological sections of the trachea and choanae, BA is visible as forming cilia-associated bacterial colonies. Loss of ciliated epithelial cells in trachea and choanae are characteristics of bordetellosis. The lamina propria may be infiltrated by heterophils, lymphocytes and plasma cells (Arp & Cheville, 1984; Saif et al., 1981). Bronchus associated lymphoid tissue tends to be hypertrophic and protrusions of lymphoid nodules into the lumen can be observed (Gray et al., 1981, 1983a; Van Alstine & Arp, 1987a).

3.1.4. Immunity and immunoprophylaxis

3.1.4.1. Immune response

Specific anti-\emph{Bordetella}-antibodies in serum and respiratory secretions play a major role in protection against \emph{Bordetella} infections and the recovery from the disease. This was shown for \emph{B. pertussis} infection in mammals (Hellwig et al., 2001) as well as BA-infection in turkeys (Arp & Hellwig, 1988; Hinz et al., 1981; Jackwood & Saif, 1980; Suresh et al., 1994). Maternal immunoglobulins of the IgG isotype are detectable in turkey poults until the age of three weeks (Suresh et al., 1994). Some authors stated that the presence of maternal BA-specific antibodies indicates an important role of the humoral immune response for the protection of young turkey poults (Arp & Hellwig, 1988; Hinz et al., 1981). Following infection, BA-specific antibodies can be detected in serum and respiratory secretions (Arp & Cheville; Jackwood & Saif, 1980; Suresh et al., 1994). BA-antibodies of the IgG, IgM and IgA isotype reach a detectable level around one to two weeks post infection (pi), peak
It was revealed by Western blot analysis that BA-antibodies are directed against at least eight bacterial proteins (Hellwig & Arp, 1990) and it was suggested that antibodies against fimbrial proteins are effective in protecting turkeys from disease development (Akeila & Saif, 1988). Several studies demonstrated that BA strains are serologically related to each other with only little antigenic variation (Boot & Hinz, 2005; Kersters et al., 1984). More recent Western blot analyses confirmed that despite minor antigenic differences, there is a relatively high conservation of the immunogenic fimbrial proteins across the investigated isolates, which are of relevance for the protective immune response against BA (Beach et al., 2012).

### 3.1.4.2. Vaccination

Preventive measures against clinical bordetellosis in turkey flocks focus on optimising housing and climate conditions and on general biosecurity measures to prevent BA-infection as well as infections with potential secondary respiratory pathogens. Where biosecurity measures are not sufficient to control BA-infection, vaccination can be part of preventive strategies. Because of the relatively high conservation of antigenic fimbrial proteins, it is expected that live-attenuated BA-vaccines, which are based on only one isolate, are capable of inducing cross-protective immunity against several BA strains (Beach et al., 2012). In European countries, no commercially produced and licenced vaccines are available, while in the USA, two live vaccines are licensed. Snick Guard (Arko Laboraties, Jewell, IA, USA) is an attenuated vaccine, for which the manufacturer recommends application via drinking water at ten and 24 days of age. Art Vax® (Merck & Co., NJ, USA) includes a chemically induced temperature-
sensitive mutant of BA. The mutant strain was demonstrated to induce moderate serum antibody titers (Burke & Jensen, 1980) and protection against clinical disease in commercial turkey flocks (Burke & Jensen, 1981). In challenge experiments, Art Vax® failed to reduce prevalence of BA in vaccinated turkeys, but was successful in mitigating clinical signs in challenged birds (Houghten et al., 1987). Following the manufacturer’s instructions, this vaccine should be applied by spraying at the first day of age, followed by a booster vaccination via drinking water application at two weeks of age. A revaccination every four to six weeks is recommended.

In countries where no suitable vaccine is commercially available, turkey flocks with clinically relevant BA-prevalence can be vaccinated with flock-specific, autologous, inactivated vaccines via parenteral application. However, it needs to be considered that turkeys are able to develop an effective immune response after BA-vaccination earliest at the age of two to three weeks (Hofstad & Jeska, 1985). Consequently, protection of turkeys under that age is not achievable by vaccination of the poults. It has been shown that vaccination of breeder hens with inactivated bacteria leads to detectable maternal antibody titers in the progeny for the first two weeks of life (Neighbor et al., 1991) and may be a potential tool for mitigating and delaying the clinical disease in challenged turkey poults (Barnes & Hofstad, 1983; Hinz et al., 1981).

A recent study, which was performed in chickens, demonstrated efficacy of a newly developed DNA vaccine which included a recombinant plasmid expressing the BA outer membrane protein A (ompA) as a protective antigen and Taishan Pinus massoniana Pollen Polysaccharides (TPPPS) as an adjuvant. The vaccine induced specific humoral and cellular immune responses against BA and protected the chickens against clinical signs following BA-challenge (Zhu et al., 2016). The working group did not investigate the effect of vaccination on colonisation with BA and spreading of the pathogen and it is not clear if the results are transferable to turkeys.
3.1.5 Diagnosis

As clinical and pathomorphological signs of a BA-infection are not pathognomonic, direct or indirect detection of the pathogen is necessary for diagnosis. BA can be isolated from organ samples or swabs from the respiratory tract. It has been shown that following BA-inoculation of one-week-old turkeys, the pathogen can be reisolated from the respiratory tract over three to five weeks pi (Cook et al., 1991). BA can be cultivated on several solid media (Arp, 1986). When cultivated for 24 h on Columbia sheep blood (CSB) agar, BA produces translucent, small, compact, and pearlike colonies of less than 1 mm diameter (Kersters et al., 1984). A second colony type with a more rough appearance has also been described (Jackwood et al., 1991). Cultivation on McConkey agar allows selection of gram-negative bacteria and a morphological differentiation from *Escherichia coli*, which is an important secondary pathogen in BA-infections (Saif et al., 1980; Van Alstine & Arp, 1988). After 24 h of incubation, colonies of BA are clear and of pinpoint size, while after 48 h, the colonies are easier to find and show a raised centre of brownish colour.

If the pathogen is isolated in pure culture, biochemical characteristics are a helpful tool for identification of BA. It reacts positive in the catalase and oxidase test and the bacteria are negative for urease reaction and nitrate reduction (Jackwood et al., 1985; Kersters et al., 1984). Frequently used methods for biochemical and physical pathogen identification are the API® 20NE®-Testkit for non-enteric Gram-negative rods (Biomérieux SA France, Marcy-L’étoile, France) and the matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Additionally, a monoclonal antibody-based latex bead agglutination test (Suresh & Arp, 1993), an indirect fluorescent antibody staining technique (Suresh, 1993) and a capillary gas chromatography assay for the detection of their cellular carbohydrate profile (Mouahid et al., 1991) have been described. Sequencing of the 16S-rRNA-gene, polymerase chain reaction (PCR) (Register & Yersin, 2005; Savelkoul et al., 1993) and real time quantitative PCR (Stenzel et al., 2017) are suitable moleculargenetic methods for the detection of BA. For the detection of BA-specific antibodies, a microagglutination test (Jackwood & Saif, 1980) and an ELISA technique have been
described (Hopkins et al., 1988). An ELISA kit is commercially available for routine diagnostics (Bordetella Avium Antibody Test Kit Turkey ProFlock®, Zoetis, NJ).

### 3.1.6 Therapy

Antibiotic treatment can reduce clinical symptoms (Van Alstine & Hofstad, 1985), especially when secondary bacterial infections are present, but usually it does not eliminate the pathogen from an infected flock and benefit of treatment is under discussion (Jackwood & Saif, 2013). It has to be taken into account that secondary bacterial or viral infections play an important role in the clinical outcome of avian Bordetellosis and that these pathogens should also be a target of treatment and preventive measures. As comparative studies revealed some diversity in antibiotic resistance of BA field strains (Beach et al., 2012; Blackall et al., 1995; Grespan et al., 2012; Mortensen et al., 1989; Szabo et al., 2015), the consideration of antibiotic resistance profile of the respective strains is important for an effective treatment. Yersin et al. found evidence that niacin application via drinking water reduces adherence of BA to the respiratory epithelium, ciliary loss and clinical signs in BA-infected turkey poults by enhancing clearance function of the epithelium (Yersin et al., 1991b).
3.2 Models for BA-infection

3.2.1 In vivo models

As young turkeys are the most relevant and susceptible host of BA (Hinz et al., 1978; Kersters et al., 1984; Saif et al., 1980), in vivo investigations on BA-infection include mainly inoculation of turkey poults in their first days of life. Several studies investigated the clinical signs and local lesions in the respiratory tract subsequently to BA-infection (Arp & Fagerland, 1987; Gray et al., 1983a; Saif et al., 1980; Temple et al., 1998; Yersin et al., 1998) and the host’s immune response to the pathogen (Fix & Arp, 1989; Suresh et al., 1994; Van Alstine & Arp, 1988). Furthermore, the influence of BA on the susceptibility for secondary infections, such as Escherichia coli were analysed (Van Alstine & Arp, 1987a, 1987c). The in vivo experiments also allowed for investigation of the influence of BA-infection on brain and heart physiology (Edens et al., 1987; Yersin et al., 1991a). Regarding the clinical outcome of the infection, effect of environmental conditions (Temple et al., 1998), of host genetic background (Sacco et al., 2000a; Temple et al., 1998) and of treatment with several substances, such as tryptophan or niacin (Edens et al., 1999; Yersin et al., 1991b), were investigated. BA field and mutant strains were characterised in their virulence characteristics (Domingo et al., 1992; Gentry-Weeks et al., 1991; Moore et al., 1994; Temple et al., 2010) and toxin production (Gentry-Weeks et al., 1988; Rhoades & Rimler, 1987; Simmons et al., 1986).

Experiments including older turkeys were performed less frequently. These studies aimed to investigate the effect of BA-infection on the pathogenesis of other viral or bacterial pathogens, such as AMPV (Cook et al., 1991; Jirjis et al., 2004). Additionally, immunogenic effects of vaccine candidates or components (Akeila & Saif, 1988; Hofstad & Jeska, 1985; Houghten et al., 1987) as well as protective effects of passive immunisation (Arp & Hellwig, 1988; Suresh & Arp, 1995a) were analysed.
To test BA strains for toxin production, some working groups used other model species, such as mice (Blackall & Rogers, 1991; Gentry-Weeks et al., 1988; Rimler, 1985; Simmons et al., 1986), Japanese quails (Simmons et al., 1986) and chickens (Gentry-Weeks et al., 1988). Chickens were additionally used to investigate the effect of immunosuppressive (Liang et al., 2013) or immunostimulating agents (Dong et al., 2016) on the immune response to BA-infection.

3.2.2 In vitro models

In infection research, in vitro alternatives to animal experiments are of scientific interest as they facilitate investigation of single aspects of pathogenesis, i.e. reactions of single host cells or tissue types. Additionally, application of these methods can reduce or replace animal experiments, which is not only legally required in many countries but also desirable and necessary from ethical perspectives.

As the epithelium of the upper respiratory tract is the target tissue of BA, tracheal organ cultures (TOCs) gained from turkey embryos are the most frequently used in vitro model for BA infection. TOCs are used for investigation and isolation of different respiratory pathogens of different host species for approximately 50 years (Cook et al., 1976; Hoorn & Tyrrell, 1969; McIntosh et al., 1967). As an organ culture model, TOCs include several epithelia-associated cell types, which represent the cell population in the respective origin animal. In contrast to the in vivo situation, the culture conditions are controllable and allow for standardisation of experiments.

TOCs prepared from tracheae of turkey embryos were used to investigate local lesions in the tracheal epithelium, which are induced by BA-infection (Gray et al., 1981; Gray et al., 1983b; Marshall et al., 1984; Van Alstine & Arp, 1987b). The role of single virulence factors on epithelial damage induction and adherence have been of particular interest in BA-research in TOCs (Shelton et al., 2002; Spears et al., 2000; Stockwell et al., 2011; Temple et al., 2010; Van Alstine & Arp, 1987b). Additionally, TOCs were used to characterise BA field and mutant strains regarding their ability to adhere to the tracheal mucosa (Grespan et al., 2012; Jackwood & Saif, 1987; Marshall et al., 1984; Temple et al., 1998), which was found to be a reliable indicator
for *in vivo* virulence of the BA strains (Temple et al., 1998). A novel culture system of tracheal explant cultures was described recently and used to investigate induction of apoptosis and nitric oxide synthase by BA (Miyamoto et al., 2011).

Further *in vitro* models were applied to investigate toxicity of BA-beta-cystathionase towards osteogenic cells (Gentry-Weeks et al., 1993; Gentry-Weeks et al., 1995) and cytotoxicity of BA in VERO cells (monkey kidney cells) (Grespan et al., 2012).

In the future, advanced tissue culture methods of the respiratory tract, such as air-liquid interface (ALI) cultures, may be used for investigation of host-pathogen interaction in BA-infection, as it has already been described for *Bordetella pertussis* (Hasan et al., 2018; Zanaboni et al., 2016).
3.3. **Aim of the study**

The aim of the present research project was to contribute to the improvement of animal health and welfare in turkey flocks by providing basic knowledge about BA-infection, which is supposed to facilitate further research on BA and its control as well as the development of innovative BA-vaccines.

In the first part of the project (1\textsuperscript{st} publication, chapter 4), we aimed to enhance understanding of the pathogenesis of BA-infection and to establish a reliable \textit{in vivo} model for BA-challenge, which is suitable for evaluating efficacy of newly developed vaccination strategies against the pathogen. The objectives of the first part were:

- Comparison of different routes and doses of BA-inoculation regarding the clinical outcome, the pathological alterations, the BA-colonisation of the respiratory tract and the BA-specific humoral immune response
- Confirmation of the reliability of the model by applying it for the evaluation of the efficacy of a formalin-inactivated vaccine against BA

The second part of the project (2\textsuperscript{nd} publication, chapter 5) focuses on characterisation of BA field strains regarding their virulence-associated properties \textit{in vitro} and their putative virulence mechanisms. The objectives of the second part were:

- Establishment of two \textit{in vitro} assays in TOCs of turkey embryos to characterise the strains regarding their ability to adhere to the tracheal epithelium and to induce ciliostasis in tracheal epithelial cells
- Comparison of BA field strains in these virulence-associated properties in TOCs
- Investigation of genetic diversity of BA field strains by cgMLST
- Identification of possible genetic factors for reduced virulence-associated properties
4. 1st publication

Establishment of a *Bordetella avium* challenge model in turkeys

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Authors’ contributions:
RK, SR and AJ designed the study. RK performed the laboratory work. RK and HP analysed the data. RK and AJ evaluated the results. RK, AJ and SR wrote the paper.
All authors read and approved the final manuscript.
Abstract

Despite the importance of *Bordetella avium* (BA) as a respiratory pathogen of young turkeys, no infection model for the evaluation of BA-vaccine efficacy is available. The objective of this study was to evaluate the influence of route and dose of infection on the establishment of a BA-challenge model. In our first experiment, 28-day-old turkeys were either inoculated oculonasally with $10^5$, $10^7$ or $10^9$ colony forming units (CFU) of BA per bird or exposed to BA by aerosol with $10^5$–$10^8$ CFU/m$^3$. The respiratory tract of all inoculated birds was BA-colonized, which was confirmed by choanal swabs and samples of trachea and lung, showing the highest prevalence in the aerosol-inoculated group. BA-specific humoral immune response was detected in the form of IgG in serum from five days post infection (dpi) and IgA in lacrimal fluid from seven dpi. In the second experiment, the model was tested in a vaccination trial. Twenty-one-day-old turkeys were vaccinated with a formalininactivated BA-vaccine intramuscularly and challenged 21 days post vaccination with $10^7$ CFU per bird oculonasally. BA-specific IgG antibodies were detected in serum and in lacrimal fluid 14 days post vaccination. As in the first experiment, secretory BA-specific antibodies of the IgA isotype were only detected in the inoculated groups from seven dpi. Despite the lack of clinical signs or pathological alterations in both experiments, vaccine efficacy was demonstrated by significant reduction in BA colonization of the trachea ($P \leq 0.05$). In our study, a reliable model for BA infection has been established and has been demonstrated to be suitable for evaluation of vaccine efficacy.
5. 2\textsuperscript{nd} publication

\textit{In vitro} characterization and genetic diversity of \textit{Bordetella avium} field strains

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\textbf{Authors’ contributions:}
RK, SR and AJ designed the study. RK performed the laboratory work for the \textit{in vitro} characterisation. HJL and MM conducted genetic analyses. RK analysed the data of the \textit{in vitro} characterisation. HJL analysed the data of the genetic investigations. RK, HP, HSL and AJ evaluated the results. RK, SR and AJ wrote the paper. All authors read and approved the final manuscript.
Summary

Bordetella avium (BA) is a respiratory pathogen of particular importance for commercially raised turkeys. Specific adherence to the respiratory epithelia and ciliostatic effects are crucial steps of the pathogenesis, but knowledge about the mechanisms and the variety of virulence in field strains is limited. In this work, we analyzed 17 BA field strains regarding their in vitro-virulence associated properties and genetic diversity. Virulence testing was done in two in vitro assays in tracheal organ cultures (TOC) of turkey embryos. The adherence assay indicated that BA field strains differ considerably in their ability to adhere to the tracheal mucosa, while the ciliostasis assay illustrated a high degree of diversity in ciliostatic effects of BA field strains. These two virulence associated properties were associated in the investigated strains, as strains with a relatively high degree of adherence also induced significant ciliostasis in the TOCs and vice versa. Three of the investigated strains displayed reproducibly lower in vitro-virulence in comparison to the other strains. Genetic diversity of BA strains was analyzed by core genome multilocus sequence typing (cgMLST). We applied a cgMLST scheme comprising 2,667 targets of the reference genome (77.3 % of complete genome). The results showed a surprisingly high degree of genetic diversity in the 17 strains, but without correlation between sequence type and virulence associated strain properties. However, the cgMLST analysis revealed that a common characteristic of all strains with less marked virulence associated properties were mutations in the putative Filamentous Hemagglutinin (FHA) protein. Amino acid sequence alignment confirmed a variation in that protein. The results from our study show that both adherence and ciliostasis assay can be used for virulence characterization of BA. Variations in the FHA protein may be responsible for reduced virulence of BA field strains.
Introduction

*Bordetella avium* (BA) belongs to the genus *Bordetella*, which contains nine species with varying host tropism. While the phylogenetically closely related species *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* are adapted to mammalian hosts, the more distantly related *B. hinzii* and *B. avium* are bird-associated. Regarding the remaining species *B. trematum*, *B. petrii* and *B. antsorpii*, host tropism, reservoir and pathogenic role have not been extensively studied yet.

BA is widely disseminated in wild and domestic birds (33, 44), but commercially raised turkeys are the most affected host for clinical disease. Mainly young turkeys display respiratory symptoms during avian bordetellosis (17, 20, 38). Upon infection, BA colonizes the upper respiratory tract and adheres highly specific to the local ciliated epithelium (14, 28, 42, 47). The local infection induces ciliostasis, apoptosis and extrusion of ciliated cells from the epithelium (28). Macroscopic and histopathologic lesions are visible in the trachea of affected birds (2, 3, 15, 39). *In vitro* experiments in tracheal organ cultures (TOCs) in combination with *in vivo* experiments suggested a strong correlation between the ability of BA to adhere to the tracheal mucosa and the ability to colonize the respiratory tracts of turkeys (25, 47). As the pathogenesis of the clinically relevant *Bordetella* species shows broad similarities and all of these *Bordetella* species have strong tropism for the ciliated respiratory tissue of their respective host (1, 42), a common genetic and functional background for virulence of the species has been suggested and genomic sequences have been subjected to comparative analysis of the species (40, 43).

Until today, two BA genome sequences are available for genomic analysis. The first published BA genome (40) is related to BA strain 197N, a spontaneous nalidixic acid-resistant variant of strain 197 which was isolated from a diseased turkey in the United States (13, 38). The second published BA genome is related to BA Nh1210, a strain isolated from cockatiels affected by lockjaw syndrome (29). In comparison to other *Bordetella* species, the BA genome is relatively short with a length of approximately 3.73 Mbp and has an overall similarity of 97% with *B. pertussis*, *B. parapertussis* and
*B. bronchiseptica* regarding the nucleotides and 75% regarding the proteins (40). A genetically close relationship in virulence associated mechanisms could merely be confirmed for the mammalian associated *Bordetella* species. These species share important mechanisms for host-pathogen interaction, including adhesins and toxins (26), whereas homologues of some of these virulence factors could not be found in BA. Pertussis toxin, which significantly contributes to the pathogenesis of whooping cough induced by *B. pertussis*, as well as adenylate cyclase, which is an important virulence factor in all mammalian adapted *Bordetellae*, are not present in BA (40). Dermonecrotic Toxin is encoded in the BA genome, but the predicted protein sequence shows only low percentage of identity to its so-called ancestor species *B. bronchiseptica* (23, 40). *Bordetella* adhesins, such as Filamentous Hemagglutinin (FHA), are located on the bacterial surface. They play an important role in the adhesion process and the colonization of the host’s respiratory epithelium (11, 48). FhaB, the preprotein of FHA, is found in BA, but has only a low percentage of sequence similarity to the FhaB of *B. bronchiseptica* (23, 40). Likewise, BA contains some exclusive FHA-like proteins in comparison to *B. bronchiseptica* (40). It has been assumed, that these differences in virulence factors represent adaptation to different host species (40). Information about diversity of BA field strains regarding virulence is very limited. Comparative studies mainly concentrate on antibiotic resistance profiles showing some variety in resistance to antibiotic substances (5, 16). Furthermore, ten BA strains isolated from cockatiels and one strain isolated from turkey did not differ in virulence associated properties, such as tracheal attachment or cytotoxic effects (16). Jackwood et al. compared different strains of *Alcaligenes faecalis*, which was previously considered to be identical with BA. They detected two subtypes of *A. faecalis* with subtype II having no pathogenic effect in turkeys and being hemagglutination negative in contrast to subtype I, which was pathogenic in poults and capable of agglutinating guinea pig erythrocytes (18). In more recent studies, it was ascertained that these non-pathogenic *A. faecalis* isolates do not represent members of the BA species. Using contemporary methods, they might be classified as *B. hinzii* (34).
The aim of the present study was to investigate diversity of BA field strains in virulence associated properties in an *in vitro* culture system of the upper respiratory tract of turkeys and to gain an overview of the genetic diversity of putative BA virulence factors. The ability to adhere to the epithelial surface and the inhibiting effect on ciliary activity in tracheal organ cultures (TOCs) are adopted as representative parameters for virulence of the strains. Finally, the results of 17 BA strains were correlated to their core genome multilocus sequence typing (cgMLST) data with a focus on potential differences in sequence types of adhesion-associated genes.
Materials and Methods

Bacterial strains and preparation of inocula

Seventeen BA strains isolated from the respiratory tract of different poultry species (Table 1) have been included in this study. The isolates were identified as BA by polymerase chain reaction (PCR) (35) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). For inoculation of tracheal organ cultures of turkeys (TOCs), BA strains were cultured for 24 h under aerobic conditions at 37.5°C on Columbia agar containing 7% sheep blood (Columbia sheep blood agar, CSB; Oxoid Deutschland GmbH, Wesel, Germany). Bacteria were suspended in prewarmed Earle’s Salt Solution (ESS; Biochrom, Berlin, Germany) and the turbidity of the suspension was measured using a densitometer (Densimat, Biomérieux SA France, Marcy-L’étoile). A suspension of McFarland-standard 3.1 was produced, which had been tested by serial dilutions to be equal to a bacterial concentration of $2 \times 10^9$ colony forming units (CFU)/ml. The bacterial suspension was diluted with 37°C warm ESS to the required concentrations of $2 \times 10^7$ CFU/ml and $2 \times 10^5$ CFU/ml. The number of CFU was verified immediately after inoculation by ten-fold serial dilutions of the inoculum and colony counting on CSB agar after an incubation time of 48h.

Preparation of tracheal organ cultures (TOCs)

TOCs were prepared from 26-day-old turkey embryos (Moorgut Kartzfehn, Bösel, Germany) as described previously (32). 0.8 – 1.0 mm tracheal rings were placed in sterile 5 ml tubes (Sarstedt, Nümbrecht, Germany) with 1 ml prewarmed media (Medium 199, Sigma-Aldrich, Steinheim, Germany) containing 5% fetal bovine serum (Biochrom, Berlin, Germany) and 1% Penicillin-Neomycin-Streptomycin Solution containing 5,000 units penicillin, 5 mg streptomycin and 10 mg Neomycin per ml (Sigma-Aldrich, Steinheim, Germany). TOCs were incubated for two to six days in an overhead shaker (Reax 2, Heidolph instruments, Schwabach, Germany) at lowest rotation speed (approximately 20 rpm) and 37.5°C. Preliminary to further treatment, ciliary activity was assessed using an inverted microscope (Zeiss, Oberkochen,
Germany) and only tracheal organ cultures with 100% ciliary activity were selected for the following experiments.

**Adherence assay**

Adherence assay was performed as previously described (47) with modifications. TOCs were washed three times with 2 ml ESS. Subsequently, medium was removed and each TOC was inoculated with 1 ml ESS with BA or without BA as negative control. For each strain and each bacterial concentration, six replicates were prepared. For each experiment, three Control-TOCs were inoculated with 1 ml sterile ESS to exclude contaminations and cross-contaminations between the experimental groups. TOCs were incubated for three hours under continuous overhead shaking at 37.5°C. The inocula were removed and each TOC was washed three times with 2 ml ESS for two minutes under continuous shaking. Subsequently, each TOC was placed in a new sterile 5 ml tube containing 1 ml PBS Dulbecco (Biochrom, Berlin, Germany) with 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany). TOCs were incubated for 1-2 h at 4-6°C and collected after 1 min mixing on a vortex mixer at highest power. Supernatants were diluted in tenfold steps in physiologic saline solution and plated out in duplicates on CSB agar. After 48h of incubation at 37.5°C under aerobic conditions, colonies were counted and the numbers of CFU per TOC were calculated. For each strain and each bacterial concentration, the assay was repeated once. Consequently, each strain and each bacterial concentration was tested in twelve TOCs. The results of these twelve TOCs, which were used per strain and bacterial concentration, were taken together for statistical analysis.

**Ciliostasis assay**

TOCs were washed three times with 2 ml ESS and inoculated with 1ml ESS with BA. For each strain and each inoculation dose, six TOCs were inoculated and each experiment was repeated once. For each experiment, three control TOCs were sham inoculated with sterile ESS to exclude contamination of the cultures and unspecific ciliostasis. The experiment was performed with an infectious dose of $10^5$ CFU/ TOC for 48h and $10^7$ CFU/ TOC for 24h as a faster progression of ciliostasis was expected.
in the TOCs inoculated with the high-concentrated bacterial suspension. TOCs were incubated for 48 or 24 hours at 37.5°C under continuous overhead shaking. Every 4h, ciliary activity of the tracheal epithelial cells was monitored semiquantitatively by using an inverted light microscope. Every TOC was divided virtually into twenty parts to estimate the proportion of ciliary activity in 5% steps.

**Library construction, genome sequencing and annotation**

For BA genome sequencing, bacterial colonies were suspended in 70% ethanol. Total DNA was isolated using the Zymo research Quick-DNA™ Fecal/Soil Microbe Kit (Zymo research, Irvine, CA). Next Generation Sequencing was conducted using the Illumina MiSeq V3 (Illumina Inc., San Diego, CA) and Ion Torrent Personal Genome Machine™ (PGM™) (Thermo Fisher Scientific, Waltham, MA) platforms. For sequencing with the MiSeq, shotgun and mate-pair libraries were both constructed for each strain using the NEBNext Ultra DNA Library Prep Kit E7370 (New England Biolabs GmbH, Frankfurt/Main, Germany) and Illumina Nextera Mate Pair Sample Preparation Kit FC-132-1001 (Illumina) according to the manufacturer’s protocols. Purification and size-selection was done with AMPure XP beads (Beckman Coulter, Brea, CA) and controlled on a Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA). Parts of the libraries were constructed using the Illumina TrueSeq DNA LT Sample Prep kit FC-121-2001 (Illumina) and sequenced using the MiSeq Reagent Kit v3 MS-102-3003 (2x 300-cycle) following the manufacturer’s protocol. For IonTorrent PGM™ sequencing, libraries were constructed using Ion Xpress™ Plus gDNA Fragment Library Kits (Thermo Fisher Scientific), amplified with OneTouch™ 2 200 (Thermo Fisher Scientific), and sequenced with Ion PGM™ 200 Sequencing Kit v2 (Thermo Fisher Scientific) chemistry on 316 chips following the manufacturer’s protocols. Prior to sequencing, libraries were inspected with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and Fragment Analyzer (Advanced Analytical Technologies). MIRA 4 (8), A5-miseq (9), SPAdes 3 (30), and CLC Genomics Workbench 8 (Qiagen, Venlo, the Netherlands) were used for initial assemblies. For generating pseudo draft genomes, the resulting contigs were submitted to CONTIGuator (12). Genes were annotated with the RAST pipeline (4).
Core genome multilocus sequence typing (cgMLST) analysis

To define a core genome for cgMLST (27), two putative BA genomes were downloaded from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/ accession numbers NC_010645 and JWMK00000000.1) as well as sequence data of 45 BA field isolates from our data bank were used. Field isolates were obtained from German and Polish poultry flocks and kindly provided by RIPAC-LABOR GmbH, Potsdam, Germany and Heidemark GmbH, Veterinary Laboratory, Haldensleben, Germany or originated from the Clinic for Poultry, University of Veterinary Medicine, Hannover, Germany. A genome-wide gene-by-gene comparison was performed using the cgMLST target definer function of the SeqSphere+ Software (Ridom GmbH, Münster, Germany) with default parameters as described previously (37) to determine the cgMLST gene set. The “hard defined core genome” approach was used and BA strain 197N (accession number NC 010645) served as the reference genome.

Amino acid sequence alignment

Amino acid sequence alignment was done with the constraint-based alignment tool COBALT for multiple protein sequences alignment (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) (31). The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for sequence homology searches.

Statistical analysis

Statistical analysis of adherence and ciliostasis assay was performed by Statistix 10.0 software (Analytical Software, Tallahassee, FL) using Kruskal-Wallis One-Way ANOVA and Dunn’s all pairwise comparison as post hoc test for the data sets of CFU and ciliary activity. $P \leq 0.05$ was considered as significant difference comparing different groups.
Results

Adherence assay

The ability of different BA strains to adhere to the tracheal epithelium was investigated in TOCs (Fig. 1). From every inoculated TOC, BA could be reisolated after the completion of the adherence assay, while control TOCs remained sterile throughout the experiments (data not shown). Following inoculation with both bacterial concentrations, strains BA 1, BA 4 and BA 10 adhered in lower median numbers compared to the other strains with BA 10 showing the lowest numbers of CFU. After inoculation with $10^5$ CFU/ml, the results ranged between $5.0 \times 10^0$ and $7.6 \times 10^3$ CFU/ml for BA 1, BA 4 and BA 10. In comparison, the strains with the highest adherence, namely strains BA 19, 17 and 13, ranged between $4.6 \times 10^3$ and $5.3 \times 10^4$ CFU/ml and TOC. The low adherence of strain BA 1, BA 4, and BA 10 was reproducible in the adherence assay with the higher concentrated inoculum of $10^7$ CFU/ml. The results ranged between $2.8 \times 10^3$ and $6.8 \times 10^4$ CFU/ml for these strains, while the strongest adhering strains in this assay, namely BA 19, BA 5 and BA 18 adhered with $1.7 \times 10^5$ to $2.1 \times 10^5$ CFU/ml and TOC.

These trends could not be supported entirely by statistical analysis as BA 10 differed significantly only from strains BA 3, BA 5, BA 8, BA 11, BA 12, BA 15, BA 17, BA 18 and BA 19 in the adherence assays with either bacterial concentration ($P \leq 0.05$). The difference to strains BA 6 and BA 13 was significant exclusively in the $10^5$ CFU/ml-assay and difference to strain BA 9 was significant exclusively in the adherence assay applying the higher bacterial concentration of $10^7$ CFU/ml ($P \leq 0.05$, $n = 12/group$). Strains BA 1, BA 4, BA 14 and BA 16 did not differ significantly from BA 10 neither in the adherence assay applying the lower nor in the adherence assay applying the higher bacterial concentration ($P > 0.05$).

Ciliostasis assay

TOCs were used to investigate the inhibiting effect of different BA strains on the functional activity of the ciliated tracheal epithelial cells (Fig. 2). Following BA inoculation with $10^7$ CFU/TOC, strains BA 3 and BA 18 induced relatively strong and
fast ciliostasis: Significant ciliostasis in comparison to the control TOCs was detected as early as four hours post inoculation (hpi), while most of the other strains induced significant ciliostasis at a later point in time, between eight and twelve hpi (Figure 2A, $P \leq 0.05$, $n = 12$/group). In comparison, relatively weak ciliostatic effect was observed after inoculation with strain BA 14, BA 19 and BA 16. Reduction in ciliary activity was significant in comparison to the controls at 16, 20 and 24 hpi, respectively. Strain BA 1, BA 4 and BA 10 did not induce significant ciliostasis in comparison to the control TOCs throughout either experiment.

The repetition of the experiment with the low-concentrated inoculum ($10^5$ CFU/TOC) generated comparable results, although the first ciliostatic effects were detected at least 4 h later than with $10^7$ CFU/TOC (Figure 2B). Significant differences to the ciliary activity of the control TOCs were observed from eight hpi (BA 12), twelve hpi (BA 17, BA 18), sixteen hpi (BA 3), twenty hpi (BA 6, BA 11, BA 15), 24 hpi (BA 13), 28 hpi (BA 5, BA 9, BA 14), 32 hpi (BA 16, BA 19) or 36 hpi (BA 1, BA 8) onwards ($P \leq 0.05$). BA 4 and BA 10 did not induce significant ciliostasis in comparison to the control group ($P > 0.05$). In control TOCs, ciliary activity remained at 100% throughout the observation time of 48 h in both ciliostasis assays. The results of adherence and ciliostasis assay coincided as the strains BA 1, BA 4 and BA 10, which showed lowest adherence numbers, did also induce weakest ciliostatic effect.

**cgMLST Analysis**

Genome-wide gene-by-gene comparison of two BA genomes downloaded from NCBI databank (accession numbers NC_010645 and JWMK00000000.1) and 45 field isolates, of which 17 had been applied in the adherence and ciliostasis assay, was performed to determine the cgMLST gene set. Whole genome sequence data of the 17 investigated strains in our study are submitted for release to NCBI databank under the Bioproject accession number PRJNA486903. Biosample accession numbers are listed in Table 1. Using BA strain 197N (accession numbers NC_010645) as a reference genome, the cgMLST Target Definer created a cgMLST scheme comprising 2,667 targets of the reference genome (77.3 % of the complete genome).
On average 99.1% cgMLST genes were called successfully from 47 BA genomes. Among the 2667 target genes, we have chosen 119 putative virulence-related genes for further analysis. The cgMLST profiles of the 17 BA strains (Table 1) which had been utilized for the *in vitro* assays in TOCs have been included in the generation of a minimum spanning tree (Fig 3). Based on the defined core genome, cgMLST and pairwise comparison of the strains revealed a minimum distance of one to 71 allelic differences between the strains. Regarding the sequence types, the analyzed BA strains appeared to cluster in at least two groups (Fig. 3): The first group (on the left-hand side of the distance tree) had at least 71 allelic differences in their sequence types in comparison to the second group. It included only five BA strains and between these five strains, the minimum distance amounts to six to 46 allelic differences. Most of the analyzed strains form the second group in the distance tree (on the right-hand side of the distance tree, Fig. 3) and show very limited variation in the sequence types of their core genomes. The lowest degree of difference, represented by variation in only one allele, was detected between strain BA 16 and BA 17 as well as between BA 16 and BA 4 (Fig. 3). The highest number of allele differences between two neighboured strains in the second group was 15.

To gain more detailed insight into the allelic diversity among isolates with different virulent abilities, we analyzed all pairwise allelic differences between isolates with weak and strong virulence associated properties in TOCs. Strain BA 4 and its next neighbour BA 16 differed in only one gene of the defined core genome despite their different virulence associated behaviour in the *in vitro* assays. The one single gene locus, which constitutes the sequence type variation between strain BA 4 and BA 16, was the locus for the putative FHA protein (BAV_RS09685). BA 1 and BA 3 differed in only two of the core genome genes. Of the two genes responsible for sequence type variation, one again was the locus of the putative FHA protein. The second gene locus was a fimbrial protein. Strain BA 10, finally, differed from its next neighbour BA 14 in ten loci. The ten loci with different alleles in BA 10 in comparison to BA 14 had been described to be related to signal transduction mechanisms, secretion proteins,
membrane genesis, flagellae, fimbriae, autotransporters and the FHA protein. Additionally, BA 10 was lacking two genes for putative fimbrial proteins.

**Amino acid sequence alignment**

We analyzed the protein sequences of the putative FHA protein (BAV_RS09685) in the 17 BA isolates. The amino acid sequence alignment is shown in the supplemental materials (Suppl. Fig. S1). In the highly conserved domains of the protein, some differences were detected in the amino acid sequences. These differences between the strains showed no direct correlation to the two virulence associated properties assessed in the TOCs. In an unknown region of the protein (beginning with amino acid 2320) the sequences of the analyzed isolates showed a higher degree of heterogeneity. A phylogenetic tree was constructed using the neighbour-joining method based on the COBALT multiple sequence alignment and the evolutionary distances were calculated by proportional (p) distance model (Fig. 4). Phylogenetic neighbour-joining tree analysis indicated that BA1 and BA10 formed a unique branch in the tree. No further correlation between the virulence associated properties and the amino acid sequence alignment of the putative FHA could be demonstrated.
Discussion
The present study indicates that ciliostasis and adherence assay in TOCs can be applied for characterization of BA strains. Ciliostasis and epithelial damage have been described to play an important role in pathogenesis of avian Bordetellosis (3, 14, 15, 28) as well as other *Bordetella*-induced diseases in various host species (1, 6, 7, 41, 42). In tracheal explant cultures, it has been demonstrated, that ciliostatic effects of BA can be demonstrated and investigated *in vitro* (28). It has been shown that there is a strong correlation between the ability of BA strains to adhere to the tracheal mucosa in TOCs on the one side, and the ability to colonize turkeys and to induce a respiratory disease on the other side (25, 47). Consequently, we hypothesize that the parameters of adherence and ciliostasis are meaningful criteria to estimate the *in vivo* virulence of BA strains. Comparative *in vivo* experiments were not performed in our study, because it is known from former studies that induction of clinical disease by BA inoculation is difficult under experimental conditions (19, 22, 38). Nevertheless, our hypothesis is supported by the results of our *in vitro* experiments. First, we could demonstrate that the ability to adhere to tracheal mucosa and the capacity to induce ciliostasis were associated to each other. Secondly, the characteristics of comparatively low adherence and weak ciliostatic effect were reproducible for two differently concentrated inocula (10\(^5\) and 10\(^7\) CFU/ml and TOC) in our study. Thirdly, strain BA 9, which was described in former studies to induce severe disease in one-day-old turkey poults (20), displayed marked virulence associated properties in the TOCs experiments.

The BA field strains investigated in our study displayed variations in their ability to colonize and damage the tracheal epithelium in the *in vitro* system of TOCs. Of the 17 strains included in our study, at least three strains displayed a lower ability to adhere to the tracheal epithelium and to reduce ciliary activity. These properties were tested in the TOC adherence assay and the TOC ciliostasis assay, respectively. Based on the results of the two assays, the three strains exhibiting the least pronounced virulence associated properties, namely BA 1, BA 4 and BA 10, were
selected for a more detailed analysis, even though they did not differ significantly from every single of the other investigated strains in every of the conducted assays.

CgMLST analysis illustrated a prominent genetic diversity of the investigated strains. The highest number of allelic differences between two neighboured BA strains in the minimum-spanning tree was 71 between strain BA 8 and BA 3. These findings indicate that there is a broad genetic diversity in field strains within the species of BA, which has not been studied before. The genetically most similar strains were BA 16, BA 17 and BA 4. Although epidemiological data are not available, these findings are remarkable as these strains had been isolated in two different years (2013, 2016, 2013), from three different host species (goose, broiler chicken, turkey (Table 1). Illustration of the cgMLST results in a minimum spanning tree (Fig. 3) conveyed an impression of BA strains clustering in two groups, which performed differently in the TOCs analyses. All of the strains in the first group showed distinct virulence associated properties \textit{in vitro}, while in the second group, strains with lower and higher virulence were mixed. Strains BA 1, BA 4 and BA 10, which were the ones with weakest or no virulence-associated properties \textit{in vitro}, were widely distributed throughout the second group of strains and had variable numbers of allelic differences to their respective closest neighbour. Consequently, a close relationship between the strains, which were found to have reduced virulence associated properties, could not surely be visualized based on cgMLST data as these strains did not form a distinct group in our study. A correlation of sequence type and \textit{in vitro} phenotype could therefore not be established.

The cgMLST analysis enabled us to identify a potential factor for differences in virulence associated properties. As one purpose of the analysis was to find a common underlying factor for low adherence rate and weak ciliostatic effect, the genes which had different sequence types in the strains with low virulence-associated properties in comparison to their next neighbour with more marked virulence associated properties, were further investigated. The only putative protein which was shown to have an allelic difference in all of the identified strains with low
virulence-associated properties (BA 1, BA 4, BA 10) in comparison to their next neighbour was the putative FHA (BAV_RS09685). The results indicate that mutations in the FHA locus are a common feature of the low adhering and weak ciliostatic BA strains and that these mutations may be to some extend responsible for lower virulence associated properties in TOCs. It has to be considered that only a selected number of genes, which were speculated to be virulence associated genes, were included in the core genome in this study. It is possible that some crucial genes for the differences in virulence associated properties were not included in the MLST analysis. Differences in the phenotype may possibly also be associated with other genetic deviations, for instance with genes coding for metabolic features. However, due to the outstanding importance of adherence for the pathogenesis of avian bordetellosis, it is reasonable to focus on genes which are related to this kind of host pathogen interaction. As we defined a core genome comprising 77.3 % of complete reference genome, the probability of covering the important genes is high. Furthermore, it is known from the literature that FHA plays an important role in the pathogenesis of *Bordetella* infections as it is the major adhesin and hemagglutinin in the mammalian-adapted *Bordetellae* (10, 21, 24, 36). It has been demonstrated in strain 197N that BA possesses an orthologue to the FHA protein in *B. bronchiseptica* (40, 43) and that FHA-negative mutants of that strain are attenuated in their ability to colonize the respiratory epithelium of the host (10, 43). Notably, the sequence similarity between *B. bronchiseptica* FHA and BA FHA is very low (23, 40) and the genetic organisations differ between these two species (43, 45). Additionally, FHA of BA seems to have a different function as it is not responsible for hemagglutination (43). It has been shown that BA possesses two species-specific genes involved in hemagglutination activity and adhesion to the tracheal epithelium (46) and it was speculated that these genes partly substitute the FHA function. As comparative studies including BA field strains are lacking so far, it is not clear whether the FHA is present and functional in virulent BA strains and if the FHA protein is of high relevance for the virulence of BA as it is the case for the mammalian-adapted *Bordetellae*. Our study confirms that mutations in the FHA locus indeed exist in BA
field strains and it suggests in accordance with other studies (10, 43) that these variations have significant impact on the virulence of the strains.

We furthermore analyzed the putative amino acid sequence of the FHA protein to overcome some limitations of the cgMLST method, which is not suitable for distinction between missense or nonsense and silent mutations. The amino acid sequence alignment revealed that the main sequence differences fall into an unknown region of the protein. Further analyses of the protein structure have to be conducted to assess if these mutations have an effect on the stability or activity of the protein and should be addressed in follow-up studies.

In conclusion, our investigations show that BA field strains vary significantly in their virulence associated characteristics. For three strains, the abilities to adhere to the tracheal epithelium and to induce ciliostasis in TOCs were significantly reduced in comparison to the other investigated strains. Furthermore, cgMLST analysis demonstrated a high variation in sequence types of BA field strains. Until now, only two sequences of BA have been available. We sequenced 17 additional BA strains and showed that the BA genome is more diverse than previously thought. We could not confirm a correlation between sequence type and virulence associated properties but identified mutations in the putative FHA as a potential candidate responsible for reduced virulence of BA field strains. Further investigations are needed to confirm the substantial role of FHA for virulence and the significance of other virulence factors for BA pathogenesis.
References


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Table 1. *Bordetella avium* isolates used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Biosample accession number</th>
<th>Host species</th>
<th>Organ of origin</th>
<th>Year of isolation</th>
</tr>
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<tr>
<td>BA1</td>
<td>SAMN09867708</td>
<td>turkey</td>
<td>trachea</td>
<td>2013</td>
</tr>
<tr>
<td>BA3</td>
<td>SAMN09869313</td>
<td>turkey</td>
<td>trachea</td>
<td>2011</td>
</tr>
<tr>
<td>BA4</td>
<td>SAMN09869314</td>
<td>turkey</td>
<td>pharynx</td>
<td>2013</td>
</tr>
<tr>
<td>BA5</td>
<td>SAMN09869315</td>
<td>turkey</td>
<td>trachea</td>
<td>2010</td>
</tr>
<tr>
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<td>SAMN09869316</td>
<td>turkey</td>
<td>lung</td>
<td>2012</td>
</tr>
<tr>
<td>BA8</td>
<td>SAMN09869317</td>
<td>turkey</td>
<td>unknown</td>
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</tr>
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<td>SAMN09869320</td>
<td>turkey</td>
<td>trachea</td>
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<td>SAMN09869321</td>
<td>turkey</td>
<td>trachea, air sacks</td>
<td>1982</td>
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<td>trachea, lung</td>
<td>1990</td>
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<td>goose</td>
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<td>SAMN09869324</td>
<td>turkey</td>
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* Bordetella avium (ATCC®35086™)
Fig. 1 Adherence assay in tracheal organ cultures (TOC). Number of adhered bacteria following inoculation with $10^7$ CFU (A) and $10^5$ CFU (B) of *Bordetella avium* per TOC. Bars represent the median of two experiments as a summary ($n=12$ strain/concentration).
Fig. 2 Ciliostasis assay in tracheal organ cultures (TOC). Ciliary activity following inoculation with $10^7$ CFU (A) and $10^5$ CFU (B) of *Bordetella avium* per TOC. Symbols represent the median of two experiments as a summary (n=12/ strain/ concentration).
Fig. 3 Minimum spanning tree based on core genome multilocus sequence typing (cgMLST) allelic profiles of 17 *Bordetella avium* isolates. Each circle represents an allelic profile based on sequence analysis of 119 virulence related genes. The strains with reduced virulence associated properties are colored in light gray, while strains with more marked virulence associated properties are colored in dark gray. The numbers on the connecting lines illustrate the number of target genes with differing alleles. Target genes differing between the strains with reduced virulence associated properties and their next neighbor are listed in the respective table.
Fig. 4 Neighbour joining tree based on amino acid sequence alignment of putative Filamentous Hemagglutinin protein (BAV_RS09685) of the 17 *Bordetella avium* isolates. Strains with reduced virulence associated properties are marked by black boxes.
6. Discussion

* Bordetella avium* (BA) is an important respiratory pathogen of commercially raised turkeys as it has considerable impact on health, welfare and productivity of these animals. There is no licensed vaccine commercially available, which fulfils the criteria of reliable protection against BA-colonisation of the respiratory tract. Additionally, knowledge about the mechanisms of colonisation and injury of the respiratory tract is very limited and insufficient for the development of novel vaccination strategies. The goal of the joint project, in which this study was embedded, was to contribute to enhancement of animal health and welfare as well as to the reduction of use of antibiotic substances in turkey flocks by facilitating the development of new and innovative vaccination strategies. For this goal, this study aims to provide a starting point by advancing basic knowledge on pathogenesis of BA-infection and BA-virulence.

In this thesis, a paper and a manuscript are included, which contain the results of the presented study. In chapter 4, “Establishment of a *Bordetella avium* challenge model in turkeys”, effects of route and dose of BA-inoculation on clinical outcome and immune response to the pathogen were investigated. Subsequently, the effect of intramuscular application of an formalin-inactivated BA-vaccine on these parameters was examined and we addressed the hypotheses that the applied vaccine protects the turkeys against BA-colonisation of the respiratory tract. We further addressed the hypothesis that the established challenge model is suitable for evaluating efficacy of BA-vaccines. In chapter 5, “*In vitro* characterization and genetic diversity of *Bordetella avium* field strains”, we used TOCs to characterise BA field strains regarding their ability to adhere to the tracheal mucosa and to induce ciliostasis and we performed cgMLST analysis to gain an overview on the diversity of virulence-associated genes in the BA strains. We addressed the hypothesis that BA field strains vary in their virulence-associated properties as well as in their virulence-associated genes and that there might be a relation between sequence types and their virulence-associated properties.
The current chapter provides a combined discussion of the two manuscripts of the study. Critical points will be addressed as well as future perspectives for research on BA.

6.1. Pathogenesis of BA-infection in naïve turkeys under experimental conditions

The first *in vivo* experiment described in chapter 4 was performed to investigate pathogenesis of BA-infection and to establish a challenge model to test BA-vaccines. To determine suitable route and dose of infection for the subsequent challenge model, we compared the pathogenesis of BA after inoculation of turkeys via oculonasal inoculation with $10^5$, $10^7$ and $10^9$ CFU/bird and aerosol inoculation. It was described that turkeys develop immune response to BA-vaccination not before two weeks of age (Hofstad & Jeska, 1985). To induce a measurable immune response in the animals, we inoculated the turkeys at 28 days of age.

The strain which was applied for the inoculation of the turkeys was isolated from a turkey flock with clinical avian bordetellosis and the strain showed virulence-associated properties *in vitro*, namely adherence to the tracheal epithelium and ciliostatic effect. Additionally, the BA strain did colonise the upper respiratory tract of the inoculated turkeys in the *in vivo* experiment. We consider the strain to be sufficiently virulent, as it has been shown that the adherence of BA to the tracheal epithelium, the colonisation of respiratory tract of turkeys and the *in vivo* virulence of BA strains are correlated (Temple et al., 1998). The parameter of colonisation enabled us to detect differences between the differently treated turkeys. Route and dose of inoculation had significant impact on the colonisation pattern. An inoculation with the low dose ($10^5$ CFU/bird) led to a low prevalence and a fast clearance of the pathogen from the respiratory tract of the inoculated animals. Inoculation with a higher dose of $10^7$ CFU/bird or $10^9$ CFU/bird, led to an increase of prevalence and a delay of clearance in comparison to the group, which was inoculated with the lower
dose of $10^5$ CFU/bird. 21 dpi, prevalence of BA was decreased in all of the oculonasally inoculated birds. These findings do not completely correspond to literature information, where a detection of BA from the nose for three weeks and from trachea and lung for five weeks post intranasal inoculation has been reported (Cook et al., 1991). However, it has to be considered that the turkeys were inoculated at a younger age of seven to eleven days post hatch in this former study.

In comparison to oculonasal inoculation, aerosol inoculation was more effective for colonisation of the birds in our study. BA disseminated quickly into the deeper regions of the respiratory tract and was detectable even in lungs of the turkeys in the aerosol group. Additionally, the clearance of the pathogen was delayed in the aerosol inoculated group. While colonisation of choanae decreased to day 21 pi in the oculonasally inoculated groups, the prevalence in the aerosol group remained at 100%. At the same time, six of seven turkeys of the aerosol group were colonised in the trachea, while in the $10^5$ group, two animals were colonised at that side and in the two other groups, no BA was detected in the tracheae. We speculate that nebulisation of the inoculum facilitates inhalation of BA into deeper regions of the respiratory tract. These findings suggest that aerosol is effective in transmission of the pathogen, which should be considered in preventive strategies.

Route and dose of inoculation did not have significant impact on the induction of disease, as the animals displayed no significant clinical signs and no macroscopic and histopathologic lesions could be detected in none of the inoculated groups. It is known from literature that young turkey poults between two and six weeks of age are particularly susceptible to the disease (Boycott et al., 1984; Hinz et al., 1978). Many studies on BA-pathogenesis, which did not pursue the goal to develop a challenge model, included experimental inoculation of turkey poults at their first day of life to induce severe clinical disease. The clinical signs were observed between one and four weeks post infection (Arp & Fagerland, 1987; Gray et al., 1983a; Saif et al., 2000a; Temple et al., 1998; Yersin et al., 1998). Experimental inoculation of older turkeys with a virulent strain may not always lead to clinical signs
under experimental conditions, because further factors, such as environmental conditions, stress and secondary infections play an important role in disease induction by BA (Jirjis et al., 2004; Saif et al., 1980). As we consider the applied challenge strain to be virulent, the lack of clinical signs after inoculation may be explained either by the age of the animals or the ideal housing conditions in our facilities and the absence of secondary pathogens. Even if BA-infection is more relevant in turkeys younger than 28 days, we consider our approach as clinically relevant, as natural infection of older turkeys may induce respiratory disease, especially if BA is combined with secondary pathogens, such as AMPV.

6.2. Humoral immunity following BA-infection or -vaccination of turkeys

In former studies, it was stated that the humoral immune response is the most relevant mechanism of protection against BA-infection (Arp & Hellwig, 1988; Hinz et al., 1981; Jackwood & Saif, 1980; Suresh et al., 1994). It was demonstrated that turkeys release BA-specific antibodies systemically as well as locally (Suresh et al., 1994). However, it has not been studied before whether vaccination of turkeys with inactivated BA-vaccines is also capable to induce systemic and local humoral immune response. Furthermore, it is an open question whether BA-specific antibodies on mucosal surfaces have a protective effect against BA-colonisation. In our in vivo experiments (chapter 4), we investigated the induction of systemic and local humoral immunity after inoculation with BA via different routes and after intramuscular application of an inactivated BA-vaccine. We additionally evaluated the protective effect of the vaccine-induced immune response by quantification of BA-colonisation in the trachea. Tracheal washings and lacrimal fluid were collected for the measurement of BA-specific local antibodies by ELISA. Route and dose of inoculation had only a slight impact on the magnitude and progress of the systemic IgG response. Turkeys inoculated oculonasally with a high dose responded more rapidly than the ones
inoculated with a lower dose and the aerosol inoculated group. This trend was also seen for IgG in tracheal washings. However, the impact of route and dose of infection was not significant for the local immune response, neither for IgA nor for IgG in lacrimal fluids and tracheal washings. The chronological development of the humoral immune response on the mucosal surface was consistent with data from former studies (Suresh et al., 1994).

Following vaccination with an inactivated vaccine, the turkeys developed a systemic humoral immune response which proceeded very similar to the response following infection. In contrast to inoculation with the virulent BA strain, vaccination with inactivated BA did not induce a detectable local IgA response in the lacrimal fluid. This finding is consistent with the literature, as it has been shown that parenteral application of an inactivated vaccine against respiratory pathogens may not induce a local IgA response in the respiratory tract (Papazisi et al., 2002; Trondsen et al., 2015). Secretory IgA antibodies are generally produced as a local response to the presence of the replicating bacteria and do not originate from the bloodstream (Conley & Delacroix, 1987; Mestecky et al., 1986). Interestingly, after challenge of vaccinated birds, the IgA immune response was even delayed in comparison to animals, which were challenged without vaccination. However, antibodies of the IgG isotype were detectable in the lacrimal fluid synchronously to IgG in serum. These findings indicate that IgG may transude passively from circulation to mucosal surfaces, which was already suggested in former studies (Suresh & Arp, 1995a, 1995b; Toro et al., 1993). These studies not only demonstrated that passive immunisation with BA-specific IgG led to the detection of IgG antibodies in the respiratory secretions, but also that these IgG antibodies have a protective effect against BA-colonisation after challenge (Suresh & Arp, 1995a, 1995b). This is confirmed by our results, as the vaccinated and challenged animals showed significantly lower bacterial numbers in the trachea than the naïve challenged turkeys ($P \leq 0.05$), although no IgA response was detectable. The reduced bacterial numbers on the respiratory epithelia of vaccinated turkeys in turn may be responsible for the decelerated local IgA response in vaccinated and challenged turkeys.
6.3. Applicability of the established model for vaccine testing

So far, no challenge model for the testing of vaccine efficacy against BA-infection has been established. Two live BA-vaccines are licensed for commercial use in the USA (Snick Guard, Arko Laboraties, Jewell, IA; and Art Vax®, Merck & Co., NJ), but no detailed investigation on the immune response and their effect on BA-colonisation have been published. For Art Vax®, it has been demonstrated that the vaccine induces moderate serum antibody titres (Burke & Jensen, 1980) and protection from clinical disease in commercial turkey flocks (Burke & Jensen, 1981), but it was stated that the vaccine is not capable to reduce prevalence of the pathogen (Houghten et al., 1987). However, the prevalence was only assessed qualitatively and no quantitative analysis of bacterial colonisation was performed. Additionally, local immunity in the respiratory tract was not investigated.

In Germany, no licensed BA-vaccine is available and turkey flocks can only be vaccinated by using flock-specific inactivated vaccines for intramuscular injection. For these vaccines, no efficacy data are available. Our study demonstrates that an inactivated vaccine, which is applied intramuscularly and which did probably not induce the local production of IgA antibodies or local cellular immune response, induced partial protection against BA-colonisation. Therefore, the use of flock-specific inactivated vaccines, which is currently the only available vaccination approach for the farmer, is a reasonable strategy to combat the disease in the field until new and better vaccines are available.

The application of quantitative analysis of BA-colonisation in the trachea as an indicative parameter for protection was a novelty in our study. Together with qualitative assessment of colonisation in choanae and lung, this method proved to be appropriate for evaluation of vaccine efficacy. While colonisation of choanae was not completely prevented by vaccination, bacterial numbers in the tracheae of vaccinated turkeys were reduced significantly \((P \leq 0.05)\). We think that this is of particular clinical
relevance as the trachea is the localisation of the major pathological changes following BA-infection (Arp & Cheville, 1984; Gray et al., 1983a; Van Alstine & Arp, 1987a, 1988).

The results of the experiment confirm that the model is suitable for efficacy testing of intramuscularly applied inactivated BA-vaccines. This kind of vaccination strategy has some limitations in application in commercial turkey flocks as the technique is labour and cost intensive and the treatment is stressful for the animals. Therefore, mucosal application of vaccines is desirable for commercial turkey flocks. Spray application for instance is feasible without handling of individual turkeys and without generating lesions in the turkeys’ skin. A second advantage of spray-application of vaccines against respiratory pathogens is that the vaccine is directly applied to the site of entry of the pathogen, which makes the protection most efficient (Nochi et al., 2018). Although we used an inactivated vaccine for intramuscular application in the challenge model, we may speculate that our model can be modified for the testing of spray or eye-drop vaccination. For the efficacy of a mucosal vaccine, induction of local and systemic immune response including the mucosa- and head-associated lymphoid tissues is necessary (Ryan et al., 2001). Therefore, a challenge model for testing of these vaccines should not only include measurement of BA-specific antibodies in respiratory secretions, but also local cellular immune response in the nasal-associated lymphoid tissue (NALT), conjunctiva-associated lymphoid tissue (CALT) (van Ginkel et al., 2012) and Harderian gland.

As BA induces severe clinical signs in especially younger turkeys which we had not included in our experiments, our approach can only provide a proof of principle for vaccine efficacy. If a vaccine should be developed which is protective for young poults, it has to be considered whether in ovo vaccination or vaccination of breeder hens could be a preventive measure against infection of poults (Barnes & Hofstad, 1983; Hinz et al., 1981; Neighbor et al., 1991).
6.4. In vitro models to investigate BA-infection

The choice of a culture system is dependent on the research question as the model must allow investigating the processes of interest.

Two important virulence mechanisms are described for BA of which the target tissue is the ciliated epithelium of the respiratory tract: The ability to adhere selectively to the respiratory epithelium (Gray et al., 1981; Miyamoto et al., 2011; Temple et al., 1998) and the ability to inhibit the ciliary activity of the epithelial cells, which is part of the clearance mechanism of the respiratory tract (Miyamoto et al., 2011; Van Alstine & Arp, 1987a). Because of the importance of these two properties for pathogenesis, we chose TOC of turkeys as a model for BA-infection. On the one hand, the adherence assay, which allows the quantification of the adhered bacteria following inoculation, was described by Temple et al. (Temple et al., 1998). On the other hand, the ciliostasis assay in TOCs has not been described for BA-infection until now, but we modified a protocol in which ciliostasis was shown after BA-inoculation of tracheal explant cultures (Miyamoto et al., 2011). Additionally, the ciliostasis assay was shown to be a very useful tool to evaluate the virulence of other respiratory pathogens of turkeys, such as Mycoplasma gallisepticum (Sid et al., 2016), AMPV (Hartmann et al., 2015; Naylor & Jones, 1994) or avian influenza virus (Petersen et al., 2012) as well as other Bordetella species in TOCs of their mammalian hosts, such as B. bronchiseptica in dog TOCs (Anderton et al., 2004) or B. pertussis in hamster TOCs (Collier et al., 1977).

As it is known from the literature that ciliostasis plays an important role in BA-pathogenesis, we aimed to compare BA strains regarding their ciliostatic effects. Our results (described in chapter 5) show that the inhibiting effect of BA on the ciliary activity was observable and reproducible in TOCs. Moreover, ciliostatic effect and adherence to the tracheal epithelium have been shown to be associated. These findings together with literature information support the assumption that the selected in vitro experiments represent the in vivo processes in a reliable way. We did not perform additional in vivo experiments with the tested BA strains. However,
comparison between \textit{in vitro} and \textit{in vivo} results had been given partly by the experiment of Temple et al. (Temple et al., 1998) who demonstrated by characterisation of several BA strains that adherence \textit{in vitro} is correlated to colonisation and disease induction in turkeys. Due to the covariation of the ability to adhere to the tracheal epithelium and the ciliostatic effect, both assays are in principle suitable for virulence testing of BA strains. In our experiments, the ciliostasis assay produced more clear results with statistical significant differences between the strains. The adherence assay in contrast could only show trends. On the other hand, the ciliostasis assay is not only very time-consuming and labor-intensive, but requires an intensive training of the person who is responsible for the assessment of the ciliary activity. Additionally, there might be intersubjective variations in the evaluation. This is why we recommend performing both assays in combination when striving for a reliable characterisation of BA strains. We claim that the combination of the two \textit{in vitro} models can reduce animal experiments for evaluation of virulence of BA strains.

6.5. \textbf{Diversity of BA field strains sequence types and virulence-associated properties}

There is only little information about the variety of BA strains regarding genotypes and phenotypes. However, knowledge about diversity of the species is important for diagnosis and particularly for evaluation of the clinical relevance of a detected BA strain in a turkey flock.

The comparison of 17 BA field strains we performed in TOCs (chapter 5) revealed that there are marked differences in the virulence-associated properties of these strains. While most of the strains induced strong and fast ciliostasis and adhered in high numbers to the ciliated epithelium of the TOCs, three strains showed significantly reduced ciliostatic effect and a trend of reduced adherence. These findings are astonishing because there is no information of this kind of strain differences in the literature. Comparative studies including a higher number of BA field strains are rare and these studies do not offer evidence of virulence differences
between strains: There are some studies which report differences between BA strains in expression of mouse lethal toxins (Blackall & Rogers, 1991; Rimler, 1985), but it is not clear, which kind of toxin was under investigation in these studies and whether the differences in toxin production are of relevance for the virulence of the pathogen in turkeys. It was reported that the ability to adhere to the tracheal mucosa (Arp et al., 1988), pathogenicity (Rimler & Simmons, 1983; Saif et al., 1980) and colony morphology (Jackwood et al., 1991; Kersteres et al., 1984) is manifested differently in BA field strains. However, it became clear in more recent studies that the strains, which had been investigated, were members of two different species, namely BA and *B. hinzii* (Register et al., 2003).

Former investigations indicated that BA strains show a high degree of similarity in outer membrane proteins and antigenic structures (Hellwig & Arp, 1990; Hellwig et al., 1988; Kersters et al., 1984). Consequently, it was suggested that the BA species is very homogenous with regard to its phenotype. This assumption is contradicted by the results of our *in vitro* experiments, as the strains investigated in our study showed differences in their virulence-associated properties. The open question remains what the underlying mechanisms are, which determine the reduced virulence-associated properties. We aimed to answer this question by analysing the genetic basis for virulence in the strains.

The BA strains analysed by cgMLST displayed diversity in sequence types of virulence-associated genes. Two previous studies contradicted the mentioned former investigations and indicated that genetic diversity among BA strains is considerable and greater than the genetic diversity within the *B. hinzii* species (Register et al., 2003; Sacco et al., 2000b). These findings were acquired by restriction enzyme analysis and ribotyping of BA and *B. hinzii* strains, but were not further investigated with regard to the effect of this diversity on virulence. A first description of the BA-genome was published by Sebaihia et al. (Sebaihia et al., 2006). They revealed that the BA-genome is relatively short and of limited similarity to the other *Bordetella* species. This suggested that knowledge about *B. pertussis* or *B. bronchiseptica*,...
cannot simply be transferred to BA. The sequences of two BA-genomes were published so far. Our study adds up to the number of 17 published BA-genome sequences. CgMLST analysis revealed a minimum distance between the BA strains of one to 71 allelic differences in virulence-related genes. In epidemiological studies conducted with several other bacterial species, zero to ten allele differences between isolates were regarded to represent closely related isolates of a species. In constrast, a distance exceeding 40 allelic differences was regarded to divide unrelated isolates (de Been et al., 2015; Ruppitsch et al., 2015; Zhou et al., 2017). The minimum spanning tree in our study was created based on 119 selected genes instead of all genes of the core genome. Therefore, a low number of one to ten allelic differences in our results does not allow the conclusion that the respective strains are closely related, but that they may show broad similarities in their virulence-associated genes. On the other hand, a higher number of 71 allelic differences between two strains suggests that these strains are divers in their virulence-associated genes and may be generally unrelated. Based on the comparison of the virulence-related genes, we find at least one cluster of closely related BA strains, including 12 out of 17 investigated strains. Between these strains, only one to 15 allelic differences were detected. Despite the obvious epidemiological unrelatedness, as the strains were isolated in different years, from different regions and from various species, these isolates are similar in their virulence-associated genes. The remaining five strains, which did not cluster in this closely related group, show a broader diversity in virulence-associated genes.

Further investigations should focus on questions about epidemiological relatedness, circulation of different sequence types in the field and conservation and evolutionary processes in virulence-associated genes in BA field strains. These questions may be of relevance for preventive strategies against BA-infection including vaccine development. Although cgMLST analysis brings along several limitations as it does not describe the quality of genetic differences, it provides a basis for further investigations. In combination with the sparse literature information, our results suggest that there is a need for further research on the genetic diversity of the BA
species and that the species is not as homogenous as previously thought. cgMLST could be a valuable tool for further investigations, as it allows high resolution of genome sequence variation as well as high reproducibility and portability (Bouchez et al., 2018; Maiden et al., 2013). For epidemiological investigations, a BA cgMLST scheme has to be developed and validated. Such a scheme can be made publicly available through genotyping platforms as it has already been conducted for *B. pertussis* (Bouchez et al., 2018).

### 6.6. Mutations in the putative FHA-protein as potentially responsible factors for reduced virulence-associated properties

Although a close relationship between virulence and sequence type was not detectable in our study, a common characteristic of the strains with reduced virulence-associated properties was identified: In comparison to their closest neighbour strains with distinct virulence-associated properties, the three strains with reduced virulence-associated characteristics had mutations in the putative FHA protein (BAV_RS09685). We speculate that these mutations may be to some extend responsible for lower virulence-associated properties. This is supported by literature information. FHA-negative mutants of an investigated BA strain were attenuated in their ability to colonise the respiratory epithelium (Spears et al., 2003). However, many aspects on the relevance of FHA for the virulence of BA strains are not investigated. In contrast to the other *Bordetella* species, FHA of BA is not responsible for the ability to agglutinate erythrocytes (Spears et al., 2003). Instead two BA-specific proteins, namely HagA and HagB, seem to be relevant for the adhesion process (Temple et al., 2010). Despite the differences of BA and other *Bordetella* species regarding the sequence of the putative FHA protein (Linz et al., 2016; Sebaihia et al., 2006), its genetic organisation (Spears et al., 2003) and its function, some researchers suggested that mutations of the FHA have an impact on the virulence of BA (Spears et al., 2003). Our findings support this hypothesis and additionally show that mutations in the putative FHA do exist in BA field strains.
Further work is needed to reveal how and to what extent structure and functionality of the protein are influenced by the mutations.
7. References


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