Callibacterum infetion in chickens

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Gallibacterium infection in chickens

-a study of taxonomy, epidemiology and bacteria-host interaction

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Ph.D. Thesis

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CONTENTS

Preface

The work outlined in the present thesis was made possible by a grant from the Danish Agricultural and Veterinary Research Council financing the project “Molecular taxonomy and pathogenesis of the family *Pasteurellaceae* of veterinary importance”, headed by Professor John Elmerdahl Olsen.

In particular, I would like to thank Magne Bisgaard for his ever inspiring and thorough supervision, although often pretty busy, never out of reach.

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Miki Bojesen

Summary

The present thesis contains eight chapters. Chapter one reviews the pertinent literature on *Gallibacterium* from previous investigations. Comparative aspects from closely related bacteria are included in areas where knowledge regarding *Gallibacterium* is lacking. These areas have provided the background for identifying the objectives listed for the present work. Chapter two provides a proposal for a new genus, *Gallibacterium*, comprising one species, *G. anatis*, and a description of two genomospecies. The proposal is based on the characterization of several strains previously reported as [*Actinobacillus*] *salpingitidis*, avian [*Pasteurella*] *haemolytica* or [*P*.] *anatis* by DNA-DNA hybridization, 16S rRNA and 16S-23S ITS sequencing, AFLP, PFGE, plasmid profiling and phenotypic characterization.

In chapter three was the prevalence of haemolytic *Gallibacterium* isolates in chicken flocks with different biosecurity levels, as well as is the likelihood of vertical transmission investigated. The prevalence was found very high in most systems, except in flocks with very high biosecurity. Vertical transmission was not demonstrated.

The genetic diversity of the isolates from two flocks from the prevalence study, were characterized by AFLP “fingerprinting” (Chapter 4). The results indicated a very high genetic similarity among isolates from the same flock, suggesting a clonal population structure with indications of niche adapted clonal lineages in the trachea and cloaca, respectively, of the same chicken.

Chapter five describes the development of an identification method based on 16S rRNA fluorescent *in situ* hybridization. The method enabled specific detection of *Gallibacterium* in infected tissue and was used for verification in the infection studies outlined below.

In chapter six, was the pathogenic potential of a possibly virulent strain of *G. anatis* identified in chapter four, investigated. This well characterized *G. anatis* strain was inoculated intravenously or intraperitoneally in normal and heterophil-depleted chickens. High mortality was recorded in the heterophil-depleted chickens inoculated intravenously. The heterophil-depleted chickens, inoculated intraperitoneally, developed a diffuse purulent peritonitis showing lesions corresponding to previous reports from natural infections in chickens.

Chapter seven describes an attempt to clone and characterize the genetic background for the haemolytic phenotype of *G. anatis.* However, the preliminary results revealed a gene, named *fnrG*, which is homologous to the *E. coli fnr*, a global transcriptional regulator able of activating the *E. coli* silent haemolysin *sheA*.

Finally, in chapter eight, the general results are discussed along with the main conclusions and future perspectives.

Sammendrag (Danish summary)

Denne afhandling indeholder ialt 8 kapitler. I kapitel 1 beskrives den bagrundsinformation som denne afhandlingen hviler på, samt målsætningerne for dette projekt. Kapitel 2 redegør for en taksonomisk undersøgelse omfattende bakterier, som hidtil har været klassifiseret som aviære [*Pateurella haemolytica*], [*Pasteurella*] *salpingitidis*, [*Pasteurella*] *anatis* og [*Actinobacillus*] *salpingitidis*. Undersøgelsen indrog teknikker som DNA-DNA hybridisering, 16S rRNA og 16-23S rRNA ITS sekventering, PFGE, AFLP, plasmid profilering, samt fænotypiske tests. Resultaterne herfra gav grundlag for oprettelsen af slægten *Gallibacterium*, samt en art, *G. anatis*, og to genomospecies. Forekomsten af *Gallibacterium* i 27 fjerkræflokke fordelt i grupper med forskellig "biosecurity", samt betydningen af vertikal transmission undersøgtes i kapitel 3. Forekomsten var meget udbredt og fandtes afhængig af "biosecurity", idet kun flokke der blev drevet under meget høje "biosecurity" standarder fandtes frie for infektionen. Vertikal transmission blev ikke påvist. Isolater fra to af de ovenstående flokke indgik i en undersøgelse af den genetiske diversitet af *Gallibacterium* i naturlige populationer, ved hjælp af hertil udviklet AFLP "fingerprinting" metode. Resultateterne viste en høj grad af genetisk similaritet iblandt isolater fra samme flok, samt indikationer på niché-adapterede klonale linier i hhv. trachea og kloaken i det samme dyr (Kapitel 4).

Udviklingen af en specifik identifikationsmetode, baseret på fluorescerende 16S rRNA *in situ* hybridisering, er beskrevet i kapitel 5. Metoden muliggør identifikation af *Gallibacterium* i inficeret væv fra værtsdyret og blev følgelig anvendt for verifikation af *Gallibacterium* infektion i studierne beskrevet herunder. Kapitel 6 beskriver infektionsforsøg baseret på en velkarakteriseret og formodet virulent *G. anatis* stamme. Høns med henholdsvis normal immunfunktion, samt høns der var heterofil-depleterede, inokuleredes via intravenøs eller intraperitoneal rute. Høj mortalitet blev observeret i de heterofil-depleterede høns, der var inokuleret med *G. anatis* intravenøst. De heterofil-depleterede høns, inokuleret intraperitonealt, udviklede en diffus, purulent peritonitis, svarende til læsioner beskrevet fra naturligt inficerede høns. Kapitel 7 beskriver et forsøg på at karakterisere det genetiske grundlag for *G. anatis'* hæmolytiske fænotype. Istedet herfor, resulterede den endnu ikke afsluttede undersøgelse i identifikationen af et gen, kaldet *fnrG*, som udviser stor homologi med *fnr* genet i *E. coli. fnr* koder for en transkribtions regulator, der bla. er fundet istand til at aktivere det såkaldte "stille" hæmolysin kaldet *sheA* i *E. coli.*

Endeligt, i kapitel 8, diskuteres og pespektiveres de overordnede resultater med inddragelse af ideer til fremtidige undersøgelser. Kapitlet afsluttes med en opsummering af hovedkonklusionerne fra dette arbejde.

Chapter 1

General introduction

Definition and historical aspects

The present thesis includes work, which has lead to formation of a new genus, *Gallibacterium*, comprising one species, *G. anatis,* and two genomospecies (Chapter 2). The genus represents bacteria previously reported as *Actinobacillus salpingitidis*, avian *Pasteurella haemolytica* and *P*. *anatis*. The new names will be used in the following, in order to avoid confusion.

Kjos-Hanssen (1950) published the first paper on bacteria, which most probably represents organisms subsequently named *Gallibacterium*. He isolated haemolytic *Pasteurella*-like bacteria from layers suffering from peritonitis and salpingitis. Since then, a number of authors have published work on what presumably represents *Gallibacterium* under other names (Greenham and Hill, 1962; Harbourne, 1962; Harry, 1962; Hinz, 1969; Mráz *et al*., 1976; Bisgaard, 1977; Gerlach, 1977; Mirle *et al*., 1991; Suzuki *et al*., 1997).

Taxonomy

Definition and concepts

Bacterial taxonomy comprises the disciplines of classifying, naming and identifying bacterial organisms (Sneath, 1984). Taxonomy, although often regarded as a very theoretic field, has some very important practical implications as it serves to secure a distinct, stabile and clear communication among microbiologist, and medical and veterinary practitioners (Christensen *et al*., 1999).

When bacteriology was established as a scientific discipline, taxonomy was based on relatively few phenotypic criteria including morphology, physical and biochemical features. Despite the gathering of large quantities of valuable phenotypic data, a rising number of cases emerged where is became clear that these methods were inadequate. Subsequently, the introduction of a number of genotypical methods, including DNA-DNA hybridization, rRNA homology and different DNA typing schemes aided in resolving bacterial systematics.

Colwell (1970) introduced the concept of combining an array of taxonomical techniques, a so called polyphasic taxonomy approach. The concept of polyphasic taxonomy relies on the combination of phenotypical and genotypical information. The integration of results from methods representing both phenotypical and genotypical studies enables delineation of taxa at different levels, allowing a more definite classification of bacteria (Vandamme *et al*., 1996).

Definition of genus Gallibacterium

The initial indications of the existence of a new taxonomically distinct group, subsequently named *Gallibacterium*, were made by Bisgaard (1982), who reported common phenotypical characters for strains classified as *A. salpingitidis* and avian *P*. *haemolytica*. At this stage

characterization was based solely on phenotypical methods, which did not permit definite phylogenetic analysis. However, subsequent DNA-DNA hybridization analysis of strains representing the heterogeneous group of *A. salpingitidis* and avian *P*. *haemolytica* supported the former indications of a genus-like cluster and indicated its close relation to genus *Actinobacillus* by demonstrating 48% DNA relatedness (Piechulla *et al*., 1985). A third group of strains, tentatively designated Bisgaard Taxon 1 (Bisgaard, 1982) and later named *Pasteurella anatis* by Mutters *et al*. (1985), was also found closely related to *A. salpingitidis* and avian *P*. *haemolytica*. However, DNA-DNA hybridization studies did not permit clustering of these two groups in a common genus (Piechulla *et al*. 1985).

The affiliation of *A. salpingitidis* and avian *P*. *haemolytica* to the family *Pasteurellaceae* Pohl 1981 was subsequently confirmed by rRNA-DNA hybridization, although, without fitting into the 7 rRNA branches proposed by De Ley *et al*. (1990). Consequently, only phenotypical similarity connected *P. anatis* (Bisgaard Taxon 1) and the group of *A. salpingitidis* and avian *P*. *haemolytica*.

It was not until attempts to solve the phylogeny of members the family *Pasteurellaceae* Pohl 1981 based on 16S rRNA sequence analysis, that *P. anatis* and *A. salpingitidis* was found to form a genus like cluster (Dewhirst *et al*., 1993). Difficulties were, however, still apparent as variability among isolates of this group at the phenotypical level hindered definition and characterization of new species.

Detection and identification Gallibacterium

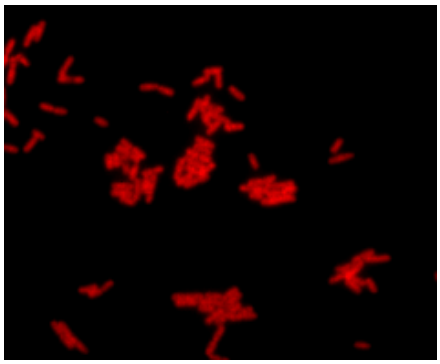
The family *Pasteurellaceae* Pohl 1981 includes taxa, which can be difficult to handle by classical methods based upon growth of the bacteria. Previously, characters like V or X-factor requirement have mainly been assigned to members of genus *Haemophilus.* However, recent work has shown that X and/or V factor-requiring species can be found throughout the family (Olsen *et al*., In press). This may diminish the likelihood of recognizing the importance of a number of taxa when primary isolation is made by conventional culture dependent methods.

Isolation and identification of *Gallibacterium* is currently dependent on cultivation of the bacterial cells. Identification of *Gallibacterium* is based on morphological characters including rod-shaped or pleomorphic cells occurring singly and in pairs, which are Gram-negative and non-motile (Fig. 1).

Gallibacterium forms grayish, semitransparent colonies, butyrous in consistence, smooth, shiny and circular with an entire margin and a size of 1-2 mm in diameter after 24 h at 37 ^{o}C. In addition, haemolytic strains produce a wide *β*-haemolytic zone (1-2 mm), (for a

comprehensive outline of phenotypical characters, see Bisgaard, 1982). Twenty-four biovars based on differences in fermentation patterns of (+)-L-Arabinose, (+)-D-Xylose, *m*-Inositol, (-)-D-Sorbitol, Maltose, Trehalose and Dextrin have been defined (Bisgaard, unpublished results). *Gallibacterium* infections do not result in pathognomonic clinical symptoms or lesions. Consequently, diagnosis can not be supported by this way.

Figure 1.
Pleomorphic shaped *Gallibacterium anatis.* Bacterial 16S rRNA was hybridized to a fluorescent Cy3™ probe. (A.M. Bojesen)



For many reasons, the lack of alternative diagnostic tools represents an undesirable situation. Firstly, traditional biochemical characterization represents laborious and time-consuming methods, which due to the phenotypically heterogonous nature of *Gallibacterium* may result in ambiguous results. Secondly, culture based methods have limitations as they do not account for viable but not cultivable cells and, furthermore, do not permit specific bacterial identification within infected tissue (Moter and Göbel, 2000). Finally, methods requiring bacterial cultivation have for *Histophilus (Haemophilus) somnus* been found less sensitive when compared to *in situ* hybridization, immunohistochemistry and various applications of PCR (Tegtmeier *et al*., 2000). Evidently, there is a need for alternative methods to identify *Gallibacterium*.

Epidemiology

Distribution and host association

Gallibacterium have been reported from many countries around the world including the European (Mráz *et al*, 1976, Bisgaard, 1977; Mirle *et al*., 1991), African (Addo and Mohan, 1984), Asian (Suzuki *et al*, 1996) and American (Shaw *et al*., 1990) continents.

Although, the chicken has been suggested to be the main host for *Gallibacterium*, these bacteria seem to be found in a wide host spectrum. Isolations have been made from several

domestic and non-domestic birds including turkeys, geese, ducks, pheasants, partridges, cattle egrets and others (Mushin *et al*., 1980; Bisgaard, 1993).

Very limited information is available on detailed epidemiological issues, as most reports are based on sporadic isolations of a few isolates, which have not permitted general conclusions. However, the occurrence of *Gallibacterium* in commercial chicken flocks was addressed by Mushin *et al*. (1980), who performed an investigation of 322 apparently healthy chicken layers originating from 23 infected flocks, where they found 97% of the birds infected. Their findings confirmed an earlier study by Bisgaard (1977) indicating that *Gallibacterium* are very common inhabitants of the respiratory tract of chickens.

Generally, prevalence proportions are likely to depend on a variety of factors where some of the more commonly recognized includes the age of the hosts, the conditions under which the hosts are kept in terms of biosecurity, and how and where the samples are taken from the chickens. However, these factors have not been studied in detail for *Gallibacterium.*

Poultry housing and management has undergone major changes since the surveys mentioned above were conducted. Especially biosecurity has been a dominating issue during the last decades, mainly as a result of national campaigns set off to exclude bacteria like *Salmonella* and *Campylobacter* in attempt to improve food safety (Anonymous, 1999). However, while increasingly comprehensive measures against microbial contamination from the environment in the existing industrial production systems have been going on for decades, yet another production form has reemerged, the organic/free-range type, as a result of increased focus on animal welfare. The organic/free-range production is characterized by a low level of biosecurity. The impact these over-all changes in the production types may have had on the prevalence and significance of *Gallibacterium* remains to be investigated.

The influence of age in terms of a “natural” age where the chickens acquire a *Gallibacterium* infection is neither known in detail, nor are the circumstances by which the birds acquire an infection with *Gallibacterium* understood. Members of the family *Pasteurellaceae* Pohl 1981 have, to our knowledge, not been shown able of genuine vertical transmission (Mannheim, 1984), and this combined with the fact that the youngest birds recorded naturally infected with *Gallibacterium* were 4 weeks old (Bisgaard, 1977), indicates horizontal dissemination as the most likely method of transmission. However, Matthes and Hanscke (1977) suggested that trans-ovarian transmission of *Gallibacterium* was possible at a low level in experimentally infected hens, indicating that vertical transmission could be a possible route. The significance of this transmission route under natural conditions is, however, unknown

and controlled studies are necessary to provide information on the importance of the various modes of transmission.

It is not clear whether infected birds are able to clear themselves from an infection or if introduction of *Gallibacterium* results in a life long infection. Matthes and Löliger (1976) performed infection studies by inoculating 10^3 cfu of *Gallibacterium* into the crop of day-old and 5-6 weeks old chickens to monitor how long the infection persisted. Interestingly, they were able to isolate *Gallibacterium* from various parts of the intestinal tract and in fewer instances from internal organs from most birds during the entire observation period lasting 8 weeks. These observations indicated that an initial infection could result in a long lasting or even life long infection. However, studies to confirm and provide further details on this point are needed as this represents a very important epidemiological issue at elucidating the infection dynamics in the chicken as well as at the flock level.

Bacterial population dynamics

Besides knowledge of the actual occurrence of *Gallibacterium* at flock and single bird level, another interesting area concerns the characteristics of the bacterial populations present here. For the time being, neither evidence nor any good tools permits assessment of clonal or genetic diversity within natural populations of *Gallibacterium*. The biovar system established by Bisgaard (unpublished results) has its limitations, as do phenotypical characterization in general for evaluation of clonal diversity in bacteria populations (Olsen *et al*., 1993). A range of techniques have, however, been developed and applied successfully for this purpose in a range of bacterial species including other members of *Pasteurellaceae*. Multilocus enzyme electrophoresis and multilocus sequence typing are both techniques that have been used extensively to evaluate bacterial population structures in general (Smith *et al*., 1993; Spratt and Maiden, 1999). Other methods, based on interpretation of DNA fragment patterns following various types of restriction enzyme digestion have been used to “fingerprint” and identify clonal lineages, which have been useful on several occasions at determining the source of disease outbreaks (Saito *et al*. 2000). Restriction enzyme analysis (REA) (MacInnes *et al*., 1990), ribotyping (Christensen *et al*., 1998), pulsed field gel electrophoresis (PFGE) (Boerlin *et al*,. 2000) and more recently amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) are examples of “fingerprinting” methods that have been widely used.

A number of parameters influence the bacterial population structure (Spratt and Maiden, 1999). However, a strong selective pressure only permitting survival of the best fitted clone resulting in a bacterial population with a high level of genetic similarity seems to represent a crucial factor (Rainey *et al*., 1993). In example, do intestinal peristalsis and the urinary flow in

urethra both serves as passive selective forces where bacteria capable of adhering to the mucosal lining have an advantage by withstanding expulsion. Uropathogenic *Escherichia coli*, which all have FimH containing fimbriae allowing them to adhere and colonize on the urinary tract epithelium (Mulvey *et al*., 2001) and adhesive *Helicobacter pylori* (Evans and Evans, 2000) are both examples of highly adapted bacterial populations. Another prominent selective force is the host immune system, which constantly targets microorganisms by different mechanisms promoting selection of adapted microbial populations.

The population structure of other members of the family *Pasteurellaceae* Pohl 1981 has been characterized. *Haemophilus influenzae* (Musser *et al*., 1985), *Pasteurella multocida* (Blackall *et al*., 1998) and *Actinobacillus pleuropneumoniae* (Møller *et al*., 1992) all exhibits a clonal population structure in natural populations. Although these three species contain commensal strains, they also accommodate highly pathogenic isolates. This is important as the actual population of a pathogenic bacterium will tend to purify and be dominated by genetically similar organisms as a consequence of sequential epidemic dissemination of the best fitted clone and subsequently elimination of the genetic variants less fitted (Achtman, 1995, 1997). In the case of *Gallibacterium*, of which the pathogenic potential needs clarification, the population structure remain unknown. Mushin *et al*. (1980) showed that a very high rate of the chickens in an infected flock sampled positive for *Gallibacterium.* However it was not stated whether that was a consequence of a single or a few contagious clones effectively spreading within the flocks or if it was caused by a high infectious pressure from a range of different clones. Evidently, a “fingerprinting” tool enabling assessment of the genetic diversity of *Gallibacterium* in natural populations would be useful to answer questions regarding the epidemiology and population dynamics for these bacteria.

Bacteria-host interactions and pathogenesis

Previous studies have shown *Gallibacterium* to be very common in the respiratory tract of apparently healthy chickens, indicating that *Gallibacterium* may be a part of the normal flora (Mráz *et al*., 1976; Bisgaard, 1977; Mushin *et al*., 1980).

Infection studies have, however, been performed in order to show if *Gallibacterium* possesses a role as a pathogen. Bisgaard (1977) made intramuscular inoculation of *Gallibacterium* in day-old chickens, but did not record subsequent disease. Another study, based on intratracheal and intramuscular inoculation of 8 week old chickens did not indicate signs of virulence either (Mushin *et al*., 1980). For the same reason it was suggested that *Gallibacterium* should be considered as a commensal of the chicken upper respiratory tract only promoting disease in cases of severely debilitated hosts.

On the other hand, *Gallibacterium* has also been reported from a number of cases, either in mixed infections or as the only microorganism present, indicating a pathogenic potential. The range of pathological lesions recorded includes salpingitis, oophoritis, peritonitis, septicemia, pericarditis, hepatitis, upper respiratory tract lesions and enteritis (Kjos-Hanssen, 1950; Greenham and Hill, 1962; Harbourne, 1962; Gerlach, 1977; Addo and Mohan, 1984; Shaw *et al*., 1990; Mirle *et al*., 1991; Suzuki *et al.*, 1997).

Although no typical lesions or syndromes have been associated *Gallibacterium* infections, several reports have demonstrated that these bacteria may be particularly associated with salpingitial and peritoneal infections (Kohlert, 1968; Gerlach, 1977; Bisgaard and Dam, 1981). These observations were substantiated further by Mirle *et al*. (1991), who isolated *Gallibacterium* as one of the most frequent single bacterial agents from hens with infections in the laying apparatus in a post mortem investigation including 496 hens.

Only a few studies have, however, succeeded in reproducing infection and disease experimentally. Gerlach (1977) performed intraperitoneally infections on day-old chickens and reported mortality rates of 85-90% within 48 hours post inoculation, using a strain obtained from diseased poultry. Another study based on inoculation of *Gallibacterium* into the crop of day-old and 5-6 weeks old chickens did only result in weak or no signs of disease when performed in normal chickens, whereas chickens infected by the same procedure, but also “cold stressed” during the infection, experienced a mortality rate at 36% (Matthes and Löliger, 1976).

Evidently, there seems to be significant differences as to virulence of different strains of *Gallibacterium*, but also characteristics related to the host seem to have a major impact on the course of infection. These characteristics suggest *Gallibacterium* as an opportunistic pathogen, which normally needs the influence of other adverse effects in order to promote disease in its host, a feature which is commonly encountered with members of the family *Pasteurellaceae* (Mannheim, 1984). However, further studies allowing insight into specific bacteria-host interactions by the use of a well characterized bacterial strain and a defined host are warranted.

Bacterial mechanisms of disease

Virulence factors have not yet been described for *Gallibacterium* and the suggestion of the existence of such mechanisms is therefore entirely a matter of speculation. However, the following outline focuses on a number of putative virulence factors described in related species, which may be a subject for future studies.

Most *Gallibacterium* isolates recovered from diseased chickens have had a haemolytic phenotype. Greenham and Hill (1962) demonstrated the haemolysis by *Gallibacterium* on nutrient agar containing bovine, horse, rabbit or chicken blood, indicating that the haemolysin(s) must work via a mechanism common to a broad range of target cells. Attempts were also made to show whether haemolysis was promoted by a soluble or bacterial cell wall associated haemolysin, but unfortunately that did not provide any definitive conclusions. Other closely related bacteria encode haemolytic toxins and especially toxins of the RTX toxin family seem to be widely disseminated among members of *Pasteurellaceae* (Kuhnert *et al*., 1997; Frey *et al*., 2002). The leucotoxin in *Mannheimia haemolytica* (Lo *et al*., 1987), ApxI-IV in *Actinobacillus pleuropneumoniae* (Chang *et al*., 1989; Frey *et al*., 1991; Schaller *et al*., 1999), and AqX from *A. equuli* (Berthoud *et al*., 2002) are all of the RTX type. Both the leucotoxin and three of the Apx toxins have been shown to be virulence factors in the pathogenesis of “shipping fever” (Stevens and Czuprynski, 1996) and porcine pleuropneumoniae (Chang *et al*., 1993; Jansen *et al.,* 1995), respectively.

Whether the haemolysin produced by *Gallibacterium* is a RTX toxin contributing to virulence remains to be clarified. It seems evident that independently of possession of a RTX toxin or not, pathogenic *G. anatis* are not exclusively relying on a haemolytic phenotype, as non-haemolytic strain have also been isolated in association with lesions, indicating that virulence as in most other bacteria is mediated by an array of factors acting in concert through different mechanisms (Heithoff *et al*., 2000). This also seems to be the case with *A. equuli*, where both haemolytic and non-haemolytic isolates have been associated with diseases in horses (Christensen *et al*., 2002).

At least some strains of *Gallibacterium* have been demonstrated with a capsule (A.M Bojesen, unpublished results). The function and significance of the capsule is not known. Capsule production has also been demonstrated and characterized in *A*. *pleuropneumoniae* (Ward and Inzana, 1997; Ward *et al*., 1998) and *P*. *multocida* (Chung *et al*., 1998; Boyce *et al*., 2000), Furthermore, Boyce and Adler (2000) demonstrated that the capsule was an important virulence factor for *P*. *multocida* during infections in mice.

Additional putative virulence factors have been suggested to be of importance in other related members of the family *Pasteurellacae* Pohl 1981. Examples include immunoglobulin proteases in *H. influenzae* capable of cleaving immunoglobulin A (IgA) and/or IgG (Reinholdt and Kilian, 1997), the enzyme superoxide dismutase in *H. influenzae* and *H. parainfluenzae,* (Kroll *et al*., 1991), and *SodC*, encoding superoxide dismutase, in *A. pleuropneumoniae* serotype 3 (Langford *et al*., 1996).

The enzyme neuraminidase has the capacity of cleaving sialic acid, also called N-Acetylneuraminate, from various sialoglycoproteins and lipids. Proteins like immunoglobulins and peptide hormones contain sialic acid residues (Gottschalk, 1960; Stryer, 1995), which is why neuraminidase has been speculated to be able to play a role in the deactivation of especially immunoglobulins targeting the bacteria. Although, there is no direct evidence for its impact on virulence (Rimler and Rhoades, 1989), neuraminidase activity has been shown for *H. parasuis* (Lichtensteiner *et al*., 1997), *H. paragallinarum* (Hinz and Müller, 1977), *P. multocida* and *M. haemolytica* (Scharmann *et al*., 1970; Straus *et al.*, 1993a,b; Straus and Purdy, 1995; White *et al.,* 1995; Straus *et al.,* 1996), where it has been speculated to take part in the pathogenesis.

As already emphasized, no virulence factors have hitherto been identified and characterized for *Gallibacterium* and the existence of any of such therefore remain a subject of speculation and will have to be addressed in future studies.

Aims and outline of the present thesis

The aims of the studies described in this thesis were firstly to provide a uniform definition of bacteria previously reported as avian [*Pasteurella*] *haemolytica* / *Actinobacillus salpingitidis* or *Pasteurella anatis*, and secondly to address some of the points outlined in the preceding introduction where important areas of understanding in epidemiology and bacteria-host interaction are lacking. As evident, only relatively little information is available on this group of bacteria and this project was therefore initiated to provide a basic understanding as well as tools for further studies of bacteria formerly known as avian [*Pasteurella*] *haemolytica* / *Actinobacillus salpingitidis* or *Pasteurella anatis.*

To achieve this we investigated the genetic relationships among avian isolates belonging to the group described above, leading to the proposal a new genus named *Gallibacterium* and a named species, *G. anatis*, and two additional genomospecies (Chapter 2). The prevalence of *Gallibacterium* in different Danish chicken production systems and the likelihood of vertical transmission between the hen and its off-spring were addressed in chapter 3. Isolates obtained from two of the flocks investigated in chapter 3 and a number of epidemiologically independent strains made the basis for the development of an AFLP genotyping method, which was used to determine the genetic diversity of *Gallibacterium* isolates at flock level and allow identification of potentially pathogenic strains. Strains from tracheal- and cloacal samples from the same bird were included, in order to, evaluate diversity of isolates from different anatomical positions in the chickens (Chapter 4).

From the results of the genetic diversity investigation, a clone, *G*. *anatis* 12656-12, associated with septicemia in two different chickens within the same flock, was identified. As this was the first remote evidence of a virulent strain, this strain was chosen for the further infection studies. Basic infection trials were attempted, however, in order to permit evaluation of the spatial distribution of *Gallibacterium* in the host tissue during infection, an *in situ* hybridization method based on a probe specific for *Gallibacterium* 16S rRNA was developed (Chapter 5). Subsequently, infection studies in chickens with a normal immune status as well as in chickens which had been immunosuppressed by heterophil-depletion were performed. Inoculation by intravenous- and intraperitoneal routes were made to assess the significance of bacterial virulence and pathological changes as a consequence of the administration route (Chapter 6). In order to elucidate which factors governs virulence in *Gallibacterium*, attempts to clone and characterize the genetic elements responsible for the haemolytic phenotype observed in many strains of *G. anatis*, were initiated. However, rather than characterizing a haemolysin, a homologue to a global regulator in *E. coli*, Fnr, which conferred a haemolytic phenotype in *E. coli* was identified. The FNR homologue in *Gallibacterium* presumably is able to activate the silent haemolysin encoded by *sheA* in the *E. coli* used as cloning host in this investigation (Chapter 7).

Finally, the over-all results are discussed and put in perspective along with a listing of the main conclusions in chapter 8.

Due to restrictions from the publishers of the journals in which the articles in chapter 2 and 4 have been published, these articles are not present in this PDF. The papers can be found in:

Chapter 2:
Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R., & Olsen, J. E. (2003). Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, "*Actinobacillus salpingitidis*" or Pasteurella anatis with proposal of *Gallibacterium anatis gen. nov.*, comb. nov. and description of additional genomospecies within *Gallibacterium gen. nov*. *International Journal of Systematic and Evolutionary Microbiology, 53,* 275-287.

Chapter 4:
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Chapter 3:
Bojesen, A. M., Nielsen, S. S., & Bisgaard, M. (2003). Prevalence and transmission of haemolytic *Gallibacterium* species in chicken production systems with different biosecurity levels. *Avian Pathology, 32,* 503-510.

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Chapter 2

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Chapter 3

Prevalence and transmission of haemolytic Gallibacterium species in chicken production systems with different biosecurity level

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Prevalence and transmission of haemolytic *Gallibacterium* species in chicken production systems with different biosecurity levels

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Summary

A stratified cross sectional study consisting of four strata of biosecurity based on production system type including organic/free-range layer-, battery-cage layer-, layer parent-, broiler parent- and broiler grandparent flocks was performed to estimate the prevalence of haemolytic *Gallibacterium* spp. Thirty birds were sampled by tracheal and cloacal swabs in each flock. A flock was considered infected when just one bird was tested positive. A total of 27 flocks were included in the study. All chickens from the broiler grandparent flocks sampled negative, whereas 28% of the broiler parents, 40% of the layer parents, 67% of the battery-cage layers and 96% of the organic/free-range chickens sampled positive. A total of 95.9% (SD ± 7.6%) of birds from infected flocks was colonized by haemolytic *Gallibacterium* species. A significantly higher number of tracheal swabs were positive compared to cloacal swabs.

The probability of vertical transfer was investigated by sampling offspring from an infected as well as a non-infected parent flock. None of the samples were found positive.

In conclusion, we showed that haemolytic *Gallibacterium* spp. were widely distributed within the Danish commercial chicken production systems. However, prevalence proportions were highly influenced by the production system and found to be significantly associated with the biosecurity level observed in the flocks. In general, flock infections resembled an “all or none” type of colonization as practically all of the chickens in infected flocks sampled positive. There was no evidence of vertical transmission of *Gallibacterium*.

Introduction

The taxonomy of organisms previously reported as [*Actinobacillus*] *salpingitidis*, avian [*Pasteurella*] *haemolytica* or [*P.*] *anatis* has recently been reinvestigated leading to the formation of a new genus, *Gallibacterium* (Christensen *et al*., 2003). The epidemiology and bacteria-host interactions of *Gallibacterium* spp. are little understood due to a lack of published literature and previous uncertainty with regard to the identification of bacteria representing this genus (Bisgaard, 1993).

The aim of the present investigation was to estimate and compare the prevalence proportions of haemolytic *Gallibacterium* spp. in chickens from different chicken production systems at different biosecurity levels. In addition, the role of vertical transmission was examined.

Bacteria belonging to genus *Gallibacterium* seem to have a wide host spectrum based on isolations from domestic as well as a range of non-domestic birds including chickens, turkeys, geese, ducks, pheasants, partridges, cattle egrets and others (Mushin, 1980; Bisgaard, 1993). Despite the seemingly cosmopolitan nature of *Gallibacterium*, previous studies have indicated that chickens are the preferred host. *Gallibacterium* spp. have been suggested to constitute a part of the normal flora in the upper respiratory and the lower genital tracts (Bisgaard, 1977). *Gallibacterium* spp. have previously been shown to be very common in layers in Denmark (Bisgaard, 1977). A study in Germany that was based on post mortem examination data from 496 hens with lesions affecting the reproductive tract, of which 50% were attributed to bacterial infections, showed *Gallibacterium* as the most common bacterial infection (23%), followed by *Escherichia coli* (21%)(Mirle, 1991). However, poultry production, including the Danish production has undergone considerable changes during the last 10-15 years primarily due to an increased focus on improved hygiene and biosecurity measures particularly associated with *Salmonella* control programs. On the other hand consumer demands towards higher welfare organic poultry products have established a basis for yet another type of production, the organic/free range system, which is characterized by a low biosecurity level. These changes may have had a great impact on the prevalence of many microorganisms, including *Gallibacterium*, as seen for poultry associated parasites (Permin *et al*., 1999).

The importance of *Gallibacterium* spp. as pathogens is not clear. Several reports have indicated that members of genus *Gallibacterium* possess pathogenic potential. Isolates have been recovered from mixed infections and in pure culture from a range of pathological lesions in poultry, including septicemia, oophoritis, follicle degeneration, salpingitis, peritonitis, enteritis and respiratory tract lesions (Kjos-Hanssen, 1950; Kohlert, 1968; Mráz *et*

al., 1976; Bisgaard, 1977; Matthes and Hanschke, 1977; Mushin *et al*., 1980; Bisgaard and Dam, 1981; Shaw *et al*., 1990, Mirle *et al*., 1991; Suzuki *et al*., 1996). Only a few experimental infections have been performed and the results were highly variable and dependent on a number of factors including the virulence of the strain, and the age and condition of the experimental birds (Harbourne, 1962; Matthes and Löliger, 1976; Bisgaard, 1977; Gerlach, 1977). The impact of *Gallibacterium* infections on modern type poultry production is therefore not known.

Consequently, we performed a cross-sectional study to determine the prevalence of haemolytic *Gallibacterium* spp. in chicken flocks representing different production systems and biosecurity levels. Chickens included in the survey were swabbed in the trachea and cloaca in order to provide an indication of site-specific differences in the prevalence of *Gallibacterium* spp. within same animal. Five different production systems were chosen in order to include different levels of biosecurity with broiler grandparent stock and organic/free range flocks representing the highest and lowest biosecurity level, respectively.

The route of transmission of *Gallibacterium* between birds is not fully understood. A previous study showed indication of trans-ovarian transfer (Matthes & Hanschke, 1977) and others have isolated *Gallibacterium* from chickens suffering from salpingitis (Gerlach, 1977; Bisgaard and Dam, 1981; Mirle, 1991) indicating that vertical transmission may play a role. However, it remains to be examined whether infected parent stock can give rise to infected offspring under natural circumstances. Therefore, newly-hatched offspring from infected parent stock were subject to bacterial examination.

Materials and Methods

Selection of flocks

The chicken flocks investigated were selected to represent different production systems. Five production systems were included representing broiler grandparents, broiler parents, layer parents, battery-cage layers and organic/free-range layers. Contact to the farmers was established by addresses provided by the Danish Poultry Council. The characteristics and biosecurity of each production system and the individual flocks were obtained on the basis of information from the farmer as well as our own observations obtained at sampling. Special attention was given to biosecurity aspects such as hygiene barriers including rules and facilities for entering the houses. The sampling took place from June 2000 to August 2001. No formal randomization was applied at the selection of flocks, although selection was attempted at random within each production system type from database registration lists. However, farmers were not always willing to participate, which is why selection had to be

made at convenience. None of the participants, at any level, had any prior knowledge of the presence of *Gallibacterium* infections during the investigation. Age, breed and disease/health status of the flocks were recorded (Table 1).

Production system characteristics

The following outline briefly summarizes the included production system characteristics, with special emphasis on biosecurity. Four biosecurity levels were established numbered 1 – 4, with 1 as the highest level and 4 as the lowest.

Organic/free-range flocks. The organic/free-range flocks were characterized as having a low biosecurity level (= 4) mainly due to missing hygiene barriers. The chickens had free access to out-door areas with potential risks of being introduced to microorganisms by contact to wild birds, other animals and humans. Sufficient hygiene management practices were also hard to establish and maintain to prevent spread of contagious organisms and carry-over from previous flocks reared in the same premises. No farm had changing rooms or showering facilities. The organic/free-range flocks were typically managed by one or two persons, who had the responsibility for all management practices in the operation. All farms were operated according to the “all in all out” principle. The flock sizes ranged from approximately 1000 to 10,000 birds.

Battery-cage layer flocks. The battery-cage layer flocks were obtained from older farms and consequently the design of the buildings was often impractical with regard to establishment and maintenance of proper hygiene barriers. All farms were operated according to the “all in all out” principle. None of the sheds had changing rooms or shower-in facilities for workers or visitors of the farm. Most farms were managed by the owner. Flock size ranged from 35- to 40,000 hens. The biosecurity level was regarded as moderate (= 3)

Layer parent flocks. The layer parent flocks represented the most diverse group with regard to production facilities. This was mainly due to the big difference in age and thereby in design of the farms included. Consequently, a substantial difference was noted in biosecurity between the flocks. Two of the flocks from this group were kept in older production facilities containing equipment mostly made out of wood and without changing rooms or shower-in facilities. They were thereby considered kept at a moderate biosecurity level (= 3). The remaining three flocks were kept in new buildings with all facilities and management practices in place to prevent introduction of microorganisms from the outside. The biosecurity level was considered high (= 2). All persons had to sign in before entering the production facilities on the farm. Subsequently visitors had to shower and change to clothes provided on the farm before entering. Key management routines were recorded on a daily basis and kept for inspection. Each flock was managed by the same few persons, each responsible for a

particular part of the daily routines, in order to minimize the number of people circulating between the contaminated and clean area. All five layer parent farms were operated according to the “all in all out” principle. The farmhouses were rodent proof with a zone kept free of vegetation to prevent rodents from residing here. Flock sizes ranged from 20- 40,000 hens.

Broiler parent flocks. The broiler parent flocks included in this study all originated from individual farms operated according to the “all in all out” principle. All farms were newly built and had high standards of biosecurity in terms of design and management practices. They were comparable with the descriptions given for the modern housing of layer parent flocks given above and were assigned as high biosecurity level (= 2). Flock sizes were from 35-40,000 birds.

Broiler grandparents. All grandparent stock originated from individual farms operated according to the “all in all out” principle. Workers as well as visitors were met by rigorous precautions to prevent introduction of disease agents from the environment. Everyone entering the premises had to provide recent evidence for a *Salmonella*-free status. In addition, they had to confirm that they had not been in contact with poultry within 48 hour prior to entry. Previously mentioned biosecurity standards were rigorously maintained including showering and complete change of clothes and footwear to that provided by the owner. The biosecurity level was kept at a very high level (= 1). Flock sizes were from 35-40,000 birds.

None of the flocks from any of the production systems included in the study had recent histories of disease problems exceeding normal levels.

Sampling

All birds older than one day were swabbed in the trachea and the cloaca using a dry, sterile cotton swab. However, the day-old chicks were only swabbed in the cloaca due to their small size. Each swab was immediately plated onto a plate of blood agar base (Oxoid), supplemented with 5% citrated bovine blood. The plates were incubated overnight at 37 ^{o}C in plastic bags. Suspect *Gallibacterium* colonies (Bisgaard, 1982) were sub-cultured on BA to obtain pure cultures. Freeze cultures were subsequently made from overnight incubated cultures in Heart Infusion Broth (HIB, Difco) at 37 ^{o}C. Seven hundred microlitres HIB were mixed with 300 µl sterile glycerol 50% and stored at -80 ^{o}C until further use.

Identification of haemolytic Gallibacterium isolates

Gallibacterium isolates were identified by being smooth and shiny, grayish, semitransparent, circular raised colonies with an entire margin and a butyrous consistency. The colonies were 1-2 mm in diameter after 24 h incubation at 37 ^{o}C and surrounded by a wide β-haemolytic

zone (1-2 mm) as defined by Bisgaard (1982) and Christensen *et al*. (2003). Two subsets of isolates were selected at random for further phenotypic characterization. One subset (130 isolates out of 810) was tested for Gram staining, motility, oxidative/fermentative, growth in Hugh & Leifsons` medium with glucose, and for urease, catalase and cytochrome oxidase activity. This enabled classification at the genus level. Another subset (70/810) was characterized using 82 phenotypic characters as described by Bisgaard *et al*., (1991). This permitted biovar assignment of the *Gallibacterium* isolates (Christensen *et al*., 2003).

Sample size

Twenty seven adult chicken flocks were included in the study. Five production systems were evenly represented by at least five flocks each. Thirty birds were selected from each flock and swabbed in the trachea and the cloaca. The birds were selected as “first seen” in the flocks where chickens were running freely in the farm house. In flocks with caged birds selection spread throughout the farm house. For the study on vertical transmission, two progeny flocks were included, one consisting of 5 week old progeny from a non-infected broiler parent flock (30 chickens sampled) and the other consisting of 300 newly hatched chickens from an infected parent flock. Assuming full diagnostic sensitivity of the detection method, sample sizes of 30 and 300 birds allow detection of infection levels greater than 10% and 5%, respectively, with 95% certainty (Martin *et al*., 1987).

Statistical analysis

The colonization status of a chicken was recorded based on tracheal and cloacal swabs. A chicken was regarded infected when the tracheal, the cloacal or both samples from the individual chicken were positive. The result of the tracheal samples was compared result of the cloaca was compared using McNemar's test for paired proportions (Altman, 1991). A flock was classified as positive if just one chicken was recorded positive in that flock. Due to the small number of flocks in each group, exact logistic regression was performed using the statistical package LogExact 4 (Metha & Patel, 2001). Odds ratios were estimated based on the estimates from the logistic regression model of the form:

$$\ln\left(\frac{P_i}{1-P_i}\right) = \mathrm{B}_i$$

where Pi = Pr(Infected flock =1| biosecurity level *i*) and B*i* is effect of the i^{th} biosecurity level (*i*=1 to 4). Difference in prevalence of infected flocks between different biosecurity levels was tested using the exact test in LogExact 4.

Results

Phenotypic characterization and identification

All presumptive *Gallibacterium* isolates (203 out of 810) recovered from primary plates and subjected to further phenotypic characterization proved to be *Gallibacterium* species. Based on these results, a percent positive predictive value of 100% was obtained by the combination of colony morphology and strong *β*-haemolysis. A negative urease test enabled discrimination between haemolytic *Gallibacterium* and haemolytic *Actinobacillus sp.* (Bisgaard Taxon 26) (Olsen *et al*., in press), the only other haemolytic, avian associated member of the *Pasteurellaceae* (Bisgaard *et al*., 1991). The extensive phenotypic characterization of 70 isolates revealed six isolates of biovar 1; two of biovar 2; 59 of biovar 4; two of biovar 7 and one of biovar 11.

Prevalence of infection

The distribution of the bacteriological results from the individual tracheal and cloacal swabs is listed in Table 1 for each of the 27 flocks along with the distribution by biosecurity level. Briefly, 13 out 27 flocks sampled positive for *Gallibacterium* species with a total of 374 infected individual hens out of 810 sampled. Comparison between colonization in tracheal and cloacal samples in the individual birds by McNemar's test revealed a significantly higher proportion of positive samples from the trachea than from the cloaca ($P < 0.01$).

Table 2 outlines the distribution of flock level infection status listed by biosecurity level. Comparison between the infection statuses of the four biosecurity levels is provided in Table 3. At a 95% level of significance, it can be seen that the prevalence proportion is significantly lower in biosecurity group 1 relative to biosecurity group 3 and 4, the prevalence is significantly lower in biosecurity group 2 relative to biosecurity group 4, whereas the remaining pairwise comparisons are statistically insignificant.

Age had no apparent influence on the prevalence of *Gallibacterium.* The effect of the breed was not taken into consideration as too many breeds were represented making analysis impossible. None of the progeny sampled, tested positive.

Table 1. *The distribution of Gallibacterium isolates from individual tracheal and cloacal swabs*

Type of birds	Biosecurity Level	Age Weeks	Chicken Breed	Positive Tracheal Samples (%)	Positive Cloacal Samples (%)	Positive Chickens[a] (%)
Broiler grandparents	1	39	Ross	0 (0%)	0 (0%)	0 (0%)
		39	Ross	0 (0%)	0 (0%)	0 (0%)
		51	Ross	0 (0%)	0 (0%)	0 (0%)
		34	Ross	0 (0%)	0 (0%)	0 (0%)
		22	Ross	0 (0%)	0 (0%)	0 (0%)
Broiler parents	2	58	Cobb	0 (0%)	0 (0%)	0 (0%)
		38	Cobb	0 (0%)	0 (0%)	0 (0%)
		42	Ross	24 (80%)	27 (90%)	28 (93%)
		46	Ross	0 (0%)	0 (0%)	0 (0%)
		19	Ross	0 (0%)	0 (0%)	0 (0%)
		22	Ross	12 (40%)	19 (63%)	22 (73%)
Layer parents	2	13	Lohmann brown	0 (0%)	0 (0%)	0 (0%)
	2	57	Lohmann brown	0 (0%)	0 (0%)	0 (0%)
	2	58	Lohmann brown	0 (0%)	0 (0%)	0 (0%)
	3	28	Isa brown	29 (97%)	23 (77%)	30 (100%)
	3	63	Isa brown	30 (100%)	23 (77%)	30 (100%)
Battery-cage layers	3	60	Lohmann brown	30 (100%)	30 (100%)	30 (100%)
		42	Isa white	30 (100%)	20 (67%)	30 (100%)
		60	Isa white	29 (97%)	17 (57%)	30 (100%)
		60	Isa white	30 (100%)	27 (90%)	30 (100%)
		52	Lohmann white	0 (0%)	0 (0%)	0 (0%)
		36	Lohmann white	0 (0%)	0 (0%)	0 (0%)
Organic layers	4	50	Isa brown	29 (97%)	29 (97%)	30 (100%)
		60	Isa brown	30 (100%)	29 (97%)	30 (100%)
		64	Isa brown	20 (67%)	25 (83%)	27 (90%)
		34	Isa brown	22 (73%)	28 (93%)	29 (97%)
		18	Isa brown	28 (93%)	20 (67%)	28 (93%)
Average age (±S.D.)		43±15.6	Average of positive[b]	26.4±5.5 (88.0±18.3%)	24.4±4.4 (81.3±14.5%)	28.8±2.3 (95.9±7.6%)
			Average of all[c]	12.3±13.9(42.4±46.3%)	10.9±12.7 (39.1±42.2%)	12.9±14.6 (43.0±48.8%)

[a] Chickens positive in either the trachea, the cloaca or at both sites. [b] Average number of positive samples in infected flocks. [c]Average number of positive samples in all flocks.

Table 2. *Distribution of infection status of Gallibacterium according to biosecurity levels*

Biosecurity level	No. positive	Total no. flocks in group
1	0 (0%)	5
2	2 (22%)	9
3	6 (48%)	8
4	5 (100%)	5

Table 3. *Association between infection status Gallibacterium and biosecurity level of 27 Danish chicken flocks, based on exact logistic regression*

Reference level (Biosecurity group)	Biosecurity level		
	2	3	4
1	1.4 [a] (0.1 - +∞) [b] P = 0.79 [c]	12 (1.2 - +∞) P = 0.033	29 (2 - +∞) P = 0.0079
2	-	9 (0.8 - 170) P = 0.089	14 (1.4 - +∞) P = 0.021
3	-	-	1.6 (0.12 - +∞) P = 0.72

[a] Numbers in cells are estimates of odds ratio. [b] 95% confidence interval. [c] Probability of similarity between biosecurity level and reference level

Discussion

The present investigation focused on five factors including production system type, biosecurity, anatomical site of isolation, breed and age, which were evaluated as risk factors for *Gallibacterium* infection. Generally, each production system was characterized by being quite uniform in terms of biosecurity, which is exemplified by the results from the broiler grandparent flocks, which never sampled positive for *Gallibacterium*. This is very likely to reflect the very high level of biosecurity maintained in this system. On the contrary, all organic flocks were infected with *Gallibacterium.* As indicated we suggest that the biosecurity rather than the production system type per se determines whether a flock is likely to be infected with *Gallibacterium* or not. This indication is even clearer when examining the system consisting of layer parent flocks where only the two flocks kept in older facilities and under less stringent hygiene management were found positive, whereas the three flocks kept in modern facilities were all negative. A similar significance of appropriate biosecurity was found in a recent analysis determining risk factors for occurrence of thermophilic *Campylobacter* spp. in Danish broiler production, where the presence of a hygiene barrier was identified as the single most important measure towards campylobacter-free broilers (Hald *et al*., 2000).

We were not able to analyse how the prevalence of *Gallibacterium* was related to the breed or type of the birds. Only very few flocks of the broiler type were infected compared to layer type flocks. This could be interpreted as a difference in susceptibility between broiler and layer type of chickens. However, the fact that the prevalence proportion in the infected broiler flocks was at a similar level as in infected layer flocks seems to argue against an association between the type of chicken and the level of *Gallibacterium* infection. Again, the general differences observed in biosecurity are more likely to account for the differences in the prevalence proportion between broiler and layer types of birds.

Our data showed that a significantly higher proportion of tracheal samples were positive than the corresponding cloacal samples from the same bird in an infected flock. This finding should be taken into consideration when designing a future study at estimating sample sizes. However, the proportion of positive samples independent of anatomical site of isolation exceeded 80% on average, making it very likely that *Gallibaterium* would be detected in either location when examining just a few chickens from an infected flock.

A common factor shown to influence results in epidemiological surveys is the age of the participants under study (Rothman and Greenland, 1998). Information on age was included in this study in order to determine whether age (length of exposure) would influence the colonization rate in the flocks. It is not clear when or how a chicken usually get infected with

Gallibacterium or if chickens are able to clear themselves from *Gallibacterium* following an infection. Age did not seem to influence the prevalence of *Gallibacterium* in the age interval used in our study.

At present no one has reported *Gallibacterium* infection from birds younger than 4 weeks of age in field studies (Bisgaard, 1977; Mushin, 1980). However, Matthes & Hanscke (1977) showed that trans-ovarian transmission of *Gallibacterium* to the egg occurred at a low level in experimentally infected hens indicating that vertical transmission was possible. None of the chicks that we examined sampled positive. In addition, 20 flocks of day-old broiler offspring has been sampled (60 chickens from each flock), as a part of another study, without isolation of a single *Gallibacterium* isolate indicating that transmission occurs by horizontal transmission (M. Bisgaard, unpublished data). Other studies on experimental infections of hens with *Salmonella* Enteritidis, known capable of vertical transfer, showed a surprisingly low proportion of infected eggs (<2%) despite evidence of ovarian and oviduct colonization in the hen (Shivaprasad *et al*., 1990; Miyamoto *et al*., 1997), suggesting that sample sizes may need to be larger in order to detect initial infection in the newly hatched chickens. It is important to note, however, that although neonatal infections with *Pasteurellaceae* have been recorded, vertical transmission does not seem to be a typical mode of transfer for members of this family (Zhao *et al*., 1993; Dritz *et al*., 1996; Zaramella *et al*., 1999), supporting the suggested insignificance of this transmission mode for *Gallibacterium* in the present study. The transmission route among birds is of importance in the perspective of designing an eradication strategy as the unlikelihood of vertical transmission leaves the efforts to be concentrated on minimising horizontal transfer, for example by means of higher biosecurity.

The identification of haemolytic *Gallibacterium* in the present study relied on bacterial cultivation on bovine blood agar plates, which at the time was the only detection method available. A previous study on *Haemophilus somnus* did, however, show direct plating of swabs from bovine lung tissue as the least sensitive detection method when compared to *in situ* hybridization, immunohistochemistry and PCR-based methods (Tegtmeier *et al*., 2000). This questions if our negative results were truly negative or an expression of lack of performance by the detection method used. However, haemolytic *Gallibacterium* spp. grew readily on bovine blood agar plates and were easily identified by their characteristic wide *β*-haemolytic zone within 24 h of incubation, hence the high positive predictive value. Furthermore, most of the positive samples showed a very high proportion of haemolytic *Gallibacterium* colonies among the total number of colonies, indicating that if *Gallibacterium* was present then the bacterial cultivation method was likely to detect it. However,

development of other preferably non-culture dependent detection methods as mentioned above should be considered as these may be more sensitive and may also allow bacterial detection *in situ*.

An alternative detection method may also help to determine the role of *Gallibacterium* as a cause of disease. The high prevalence in apparently healthy chickens has not been associated with a high number of reports of *Gallibacterium* from diseased chickens in Denmark. Whether this is because *Gallibacterium* is only rarely implicated in disease or because it is not being detected remains to be investigated. The gross pathological lesions of salpingitis/peritonitis, a typical manifestation of *Gallibacterium* infection (Bisgaard, 1977; Gerlach, 1977; Mirle *et al*., 1991), cannot easily be distinguished from a range of other Gram negative pathogens, especially *E*. *coli*, which is regarded as the most prevalent cause of this type of disease (Bisgaard & Dam, 1981). However, in cases where diagnosis is established solely by characteristics of the pathological lesions, *Gallibacterium* is not likely to be held responsible, which is why the importance of these bacteria may be underestimated. Detailed post mortem investigations including natural cases of peritonitis/salpingitis carried out as soon as sick or dead birds are observed to prevent possible overgrowth by other bacterial species, may reveal a more prominent role for *Gallibacterium* as a pathogen.

In conclusion, haemolytic *Gallibacterium* spp. were very prevalent in the Danish chicken production systems that were characterized as having low to moderate biosecurity levels, indicating that lesser biosecurity is a major risk factor for obtaining a *Gallibacterium* infection. Almost all individuals in an infected flock were infected, with a high number of both tracheal and cloacal samples being positive. The colonization rate in trachea was slightly higher than in the cloaca. Finally, prevalence was not related to age in the age interval included in the study and we did not provide evidence for vertical transmission suggesting horizontal transfer as the typical mode of transmission between birds.

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Chapter 4

Genetic diversity of *Gallibacterium anatis* isolates from different chicken flocks

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Chapter 5

Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA *in situ* hybridization

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Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA *in situ* hybridization

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Abstract

Gallibacterium has recently been included as a new genus of the family *Pasteurellaceae* Pohl 1981, which encompasses bacteria previously reported as *Pasteurella anatis*, [*Actinobacillus*] *salpingitidis* and avian *Pasteurella haemolytica*-like organisms. Identification has, so far, exclusively relied on phenotypical characterization. Here we present a method based on a Cy3™ labelled *in situ* hybridization probe targeting 16S rRNA to allow specific detection of bacteria belonging to the genus *Gallibacterium*. The probe, GAN850, showed no cross-reactivity to 25 other poultry associated bacterial species including members of the families *Pasteurellaceae*, *Enterobacteriaceae* and *Flavobacteriaceae,* when evaluated by whole cell hybridization. The probe was further evaluated by hybridization to formalin-fixed spleen and liver tissues from experimentally infected chickens, in which it proved useful for detection of *Gallibacterium*. Additionally, spatial distribution and host-cell affiliation of *Gallibacterium*, at various time points during the infection process, was possible. In conclusion, the *in situ* hybridization technique described may be of use as a diagnostic tool as well as for studies to elucidate the pathogenesis of *Gallibacterium* infections in chickens.

Introduction

Bacteria previously classified as [*Actinobacillus*] *salpingitidis, Pasteurella haemolytica*-like or *Pasteurella anatis* have recently been reclassified and relocated into a new genus, *Gallibacterium,* of the family *Pasteurellaceae* Pohl 1981 (9). Presently, the genus includes a single species, *G. anatis,* in addition to two genomospecies.

The nature of *Gallibacterium* infections is poorly understood. The bacteria has been suggested to constitute a part of the normal flora of the upper respiratory as well as in the lower genital tract of chickens (4,22,23). A recent investigation, including clinically healthy chickens from different Danish layer production systems, showed that haemolytic *Gallibacterium* were highly prevalent in birds from production systems with moderate/low biosecurity (6a). Mirle *et al*., (19) examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs, whereas, the second most prevalent agent, *E. coli,* was isolated in 21% of the cases. In addition, others have isolated *Gallibacterium* in pure culture from various pathological lesions, including salpingitis, oophoritis, peritonitis, pericarditis, hepatitis, enteritis, upper respiratory tract lesions and septicaemia (1,5,14,15,19,25-28). Evidently, the mechanisms behind the pathogenic potential of *Gallibaterium* remain to be elucidated.

The lesions reported induced by *Gallibacterium* are not pathognomonic and the detection and identification are dependent on classical isolation and identification procedures, including phenotypical characterization (9). Genus *Gallibacterium* phenotypically represents a heterogeneous group and appropriate phenotypical characterization involves laborious and time-consuming methods, which may also give ambiguous results due to variable outcomes. This in turn leads to difficulty in interpretation of genus and species designations from some earlier studies, where only relatively few phenotypical characters have been investigated (6). Additionally, *Gallibacterium*, as a cause of salpingitis and/or peritonitis, may be underestimated due to the aforementioned limitations of available diagnostic methods.

Establishment of an alternative, more accurate and reliable detection method is warranted.

Consequently, the aim of the present study was to develop a genotypic identification method allowing specific detection of *Gallibacterium* in the host.

Rapid and specific identification of individual bacterial cells can be achieved with the fluorescent *in situ* hybridization technique (FISH) (11), which is based on fluorescent labelled oligonucleotides complementary to bacterial 16S rRNA. This poses advantages compared with traditional culture based methods, as it is not restricted to live or intact cells making it suitable for the detection of viable non-culturable cells, and other fastidious organisms difficult to culture outside their natural habitat. FISH has been applied for sensitive detection

of microorganisms *in situ* and has been used to reveal bacteria-host interactions at the cellular level (3). The method has proved to be a valuable tool in elucidating pathogenesis and spatial distribution of a range of different bacteria, including *Pasteurella multocida* in chicken and porcine tissues (17); *Haemophilus somnus* in bovine lung tissue (29); *Brachyspira hyodysenteriae* and *pilosicoli* in porcine intestinal tissue (7).

In this study, we demonstrate a detection method based on a Cy-3™ labelled oligonucleotide probe specific for *Gallibacterium*. The specificity of the probe was confirmed by negative hybridization signals with 25 other poultry associated bacterial species, including members of the families *Pasteurellaceae*, *Enterobacteriaceae* and *Flavobacteriaceae*. Furthermore, the probe was shown to be specific and able to resolve spatial distribution of *Gallibacterium* spp. in splenic and liver tissue from experimentally infected chickens.

Materials and Methods

Bacterial strains and media

Thirty-three bacterial strains were included in the study (Table 1). Bacteria were stored at –80 ^{o}C and cultivated overnight on blood agar base (Oxoid, Hampshire, UK) with 5% citrated bovine blood. Single colonies were cultured in Brain Heart Infusion broth (Oxoid) with shaking, at 37 ^{o}C. Bacterial concentrations and growth phase were estimated by optical density (OD_{600}) prior to fixation.

Fixation of bacterial cells

One hundred microlitres of cultured bacterial cells in logarithmic growth phase were fixed in 800 µl of 1 x phosphate-buffered saline (PBS) (Life Technologies, Taastrup, Denmark) with 4% paraformaldehyde (Sigma, St. Louis, Mo., USA) and incubated for 1 h at 37 ^{o}C. The bacteria were pelleted by centrifugation at 5000 *g* for 5 min, washed in 500 µl, 1 x PBS with 0.1 % [vol/vol] Nonidet P-40 (Sigma Chemical, St. Louis, Mo., USA), and resuspended in a 1:1 mixture of storage buffer (20 mM Tris-HCl [pH=7.5] and 96% ethanol). Fixed cells were stored at –20 ^{o}C until use.

Oligonucleotide probes

Sequences of 16S rRNA, from 14 taxa of the family *Pasteurellaceae* Pohl 1981, obtained from GenBank were aligned with Pileup (Wisconsin Sequence Analysis Package, GCG, Madison, USA) (Table 2). A *Gallibacterium* specific oligonucleotide probe, GAN850, 5´-TTGCTTCGAGAGCCATAC-3´ and its complementary, non-GAN850, 5´-GTATGGCTCTCGAAGCAA-3´ were selected for further experiments. The uniqueness of the probe sequence was confirmed by BLASTn search (2). The eubacterial probe, EUB338, 5´-GCTGCCTCCCGTAGG-AGT-3´ (3) was included as a control. The genus and eubacterial

probes where synthesized, monolabelled at the 5´-end with Cy3™ and Cy5™, respectively, and purified by high-pressure liquid chromatography (TagC, Copenhagen, Denmark).

In situ hybridization of bacterial cells

Prior to hybridization, fixed bacterial cells were bound to poly-L-lysine (Sigma, St. Louis, USA) teflon coated slides (Novakemi AB, Enskede, Sweden) and dehydrated by sequential washes in 70 and 96% ethanol (3 min each). Ten microlitres of hybridization buffer (15% [vol/vol] formamide, 20 mM Tris, pH 7.0, 0.9 M NaCl, 0.1% [vol/vol] SDS) and 5 ng of probe were applied to each well followed by hybridization in a humidified chamber. The fluorescence signal intensity was evaluated for all strains hybridized with the probes GAN850, non-GAN850 and EUB338 at 37^{o}C; with the probes GAN850 and EUB338 at 42^{o}C, and with GAN850 at 47^{o}C. The duration of the hybridization was at least 1 h. Slides were then washed in hybridization buffer in a Coplin jar for 10 min at hybridization temperature and subsequently transferred to a washing buffer containing 20 % (vol/vol) formamide for 10 min at hybridization temperature. The slides were finally rinsed in MilliQ-water and air-dried in the dark. All hybridizations were carried out at least twice.

Preparation of histological sections for hybridization

Liver and spleen tissue originated from chickens experimentally infected with *G. anatis* strain 12656-12 as described in (A.M. Bojesen, O.L. Nielsen, J. P. Christensen and Magne Bisgaard, submitted for publication). Briefly, fifteen weeks old chickens were inoculated with *G. anatis* by intravenous or intraperitoneal routes. Chickens inoculated with saline were included as controls. Both, heterophil-depleted (5-Fluorouracil treated) and chickens with a normal immune status (saline treated) were infected with 10^7 cfu. Chickens were killed and tissue specimens were collected at 3, 12 and 24 h post inoculation. Tissue was immediately transferred to 10% phosphate-buffered formalin for fixation for 24 h followed by dehydration and embedding in paraffinwax prior to preparation of 3 to 4 μm thick cross sections. Sections were mounted on adhesive slides (Super Frost/Plus, Menzel-Gläser, Germany).

In situ hybridization of tissue sections

Tissue sections were deparafinized by xylene and dehydrated twice with 99% ethanol for 3 minutes at each step, following air drying. The hybridization and washing steps were essentially performed as with the bacterial samples except of the addition of 100 μl of hybridization buffer and 200 ng probe per tissue section. Histochemical staining of the splenic tissue was performed using haematoxylin-eosin stain (HE) for histopathological interpretation. A coverslip was mounted on hybridised slides with application of a few drops of non-fluorescent paraffin oil on the tissue sections.

Microscopy and image analysis

The slides were examined using a Zeiss filter 15 or 26 for visualisation of Cy3™ and Cy5™, respectively, by an Axioplan II epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence with a 100 W mercury lamp. A FITC/Cy3™ double filter (Chroma Technology Corp, Brattleboro, VT, USA) was used in cases where background staining of tissue as well as fluorescence from probe GAN850 was desirable, in order to attain spatial distribution of the bacterial cells. Tissue sections stained with HE were examined by light microscopy. Images were acquired with a Zeiss AxioCam digital camera.

Results

Specificity of the probe sequence and optimisation of hybridization conditions

A region corresponding to base positions 847 to 864 in the *E. coli* 16S rRNA gene sequence identified at least three mismatches separated *Gallibacterium* from 12 closely related members of the family *Pasteurellaceae* (Table 1). Database search in GenBank using BLASTn with the *Gallibacterium* specific sequence did not show high similarity to other non-*Gallibacterium* species, confirming the uniqueness of the probe.

Table 1. Sequence alignment of probe Gan850 and 16S rRNA sequences from related bacterial taxa in the family *Pasteurellaceae*

Bacterial species	**16S rRNA sequence**[a,b]	**Accession number**	**Reference**
Probe GAN850	3´ CATACCGAGAGCTTCGTT 5´		This study
Non - Probe GAN850	5´ GTATGGCTCTCGAAGCAA 3´		This study
G. anatis (*P. anatis*)	5´ . . . . . 3´	AF228001	(9)
G. genomospecies 1(*A. salpingitidis*)	C.-	AF228015	(9)
Bisgaard Taxon 3 biovar 1	. . T . . . TC	AY172724	(10)
Bisgaard Taxon 32	-TC . . . . . T.	AY172729	(10)
Bisgaard Taxon 2	GAT . . . TC . . . . . T.	L06078	(12)
Bisgaard Taxon 3	ACT . . . TC . . . . . T.	L06079	(12)
Bisgaard Taxon 26	TC- . . . TGCC . . . . . T.	AF224284	(24)
P. multocida subsp. *multocida*	C.- . . . GCCC . . . . . T.	AF294410	(16)
P. langaa	C- . . . . . GCCC . T . . . T.	M75053	(12)
A. lignieresii	TC- . . . TGCCC . . . . . T.	M75068	(12)
P. avium	C- . . . . TGCCC . T . . . T.	M75058	(12)
P. gallinarum	C- . . . . TGCCC . T . . . T.	M75059	(12)
P. volantinum	C- . . . . TGCCC . T . . . T.	M75070	(12)
P. sp. A	GG . . . C . CGTA . CTA . TT	M75055	(12)

[a] Dot (.) indicates base similarity to the complementary probe sequence.
[b] Hyphen (-) indicates a deletion in the sequence.

The specificity of probe GAN850 was tested on pure-culture of related organisms, which could pose a differential diagnostic problem. A strong fluorescent signal was detected from *Gallibacterium* spp. (Fig. 1A), whereas no or minimal detectable signals were obtained with the 24 non-*Gallibacterium* strains included (Table 2). The reverse and complementary probe, non-GAN850, did not shown any signal upon hybridization on any of the included strains (data not shown). Hybridizations with the eubacterial probe, EUB338, on all the included strains showed a signal intensity corresponding to the level observed with GAN850 hybridized with members of genus *Gallibacterium*. (Fig. 1B).

The signal intensity became slightly weaker when the hybridization temperature was raised from 37^{o}C to 42^{o}C and to 47^{o}C, but did not change the over all specificity of the probe GAN850 (data not shown). Alteration of the hybridization time between 1h and 24h did not influence hybridization specificity or signal intensity of GAN850.

Histological findings and tissue hybridization with fluorescent probes

HE staining of splenic tissue from IV inoculated chickens showed basophilic microcolonies and bacterial aggregates, primarily in the ellipsoids surrounding the penicilliform capillaries (Fig. 1C). Necrotic spleenic cells and eosinophilic material was also identified in the ellipsoids. Spleen tissue from non-infected controls was without detectable lesions (data not shown).

A strong hybridization signal was obtained with probe GAN850 in tissue sections from infected chickens. The signal distribution corresponded to the microcolonies and basophilic aggregates of bacteria as seen in the ellipsoids in the HE stained spleen sections (Fig. 1D). Single bacteria could be visualized by HE staining and GAN850 hybridization (Fig. 1E) in the liver tissue sections from the IP inoculated chickens, where bacteria were apparent in the exudates covering the serosal surface of the livers. Tissue hybridization with the eubacterial probe, EUB338, always revealed a similar pattern as that obtained with GAN850 on infected chicken tissue (data not shown), whereas hybridization with the complementary probe, non-GAN850, was negative in infected as well as non-infected control tissues (Fig. 1F).

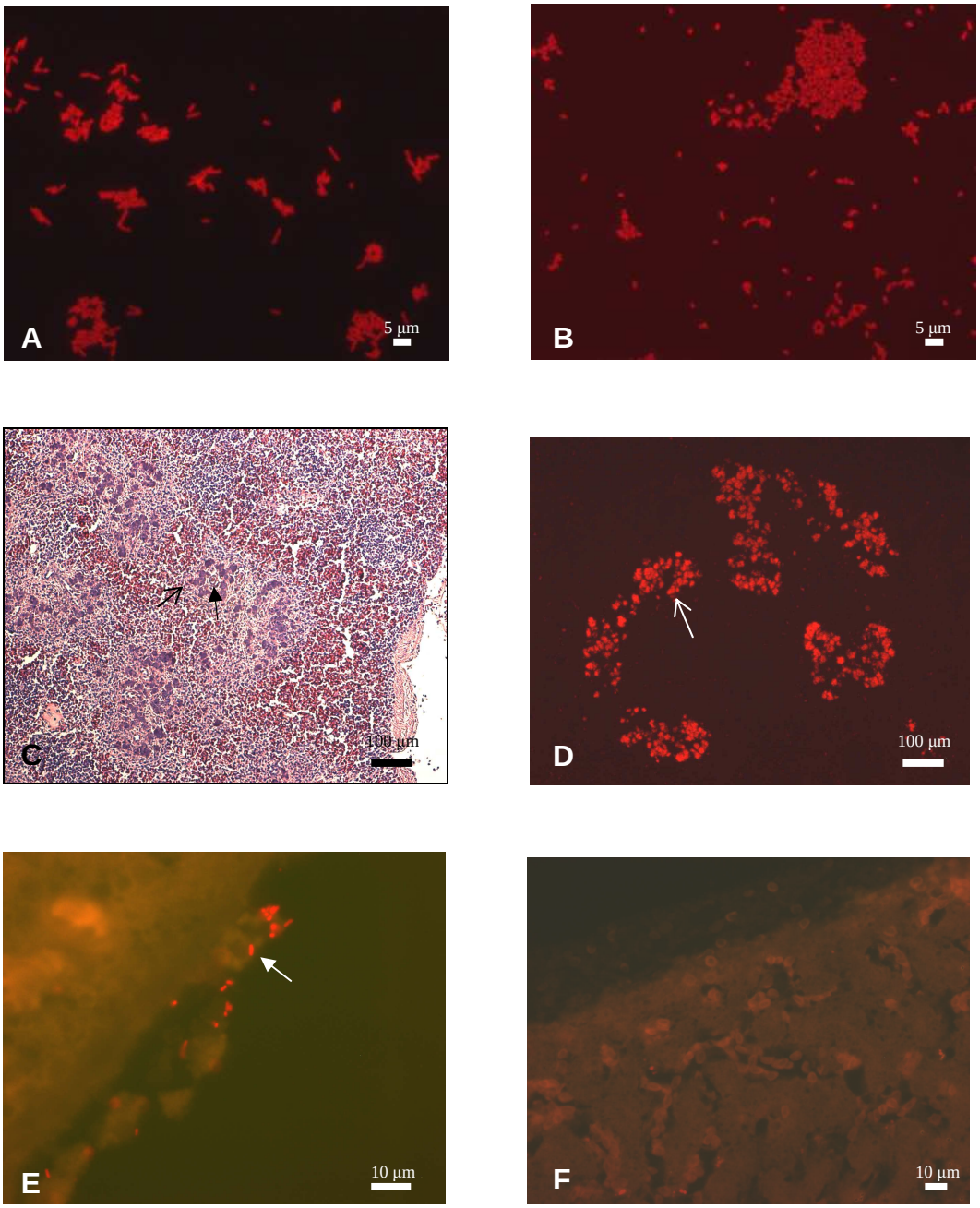
Table 2. Bacterial taxa included in Gan850 probe specificity testing on whole cells.

Bacterial strain[a]	Bacterial taxon	Animal source	Hybridization signal[b]
NCTC11413^T	*Gallibacterium anatis* (*Pasteurella anatis*)	Duck	+
CCM5976	G. genomospecies 2 (*Actinobacillus salpingitidis*)	Chicken	+
34346 Ovary	*G. anatis* (*A. salpingitidis*)	Chicken	+
10672-9	*G. anatis* (*A. salpingitidis*)	Chicken	+
12656-12 Liver	*G. anatis* (*A. salpingitidis*)	Chicken	+
24K10	*G. anatis* (*A. salpingitidis*)	Chicken	+
CCM5975	*G. anatis* (*A. salpingitidis*)	Chicken	+
Hom 69	*G. anatis* (*A. salpingitidis*)	Chicken	+
CCM5974	*G.* genomospecies 1 (*A. salpingitidis*)	Chicken	+
NCTC4189^T	*Actinobacillus lignieresii*	Cow	−
ATCC 29546^T	*Pasteurella avium*	Chicken	−
NCTC11296^T	*P. gallinarum*	Chicken	−
NCTC10322^T	*P. multocida* subsp. *multocida*	Pig	−
NCTC10204^T	*P. multocida* subsp. *gallicida*	Bovine	−
NCTC11411^T	*P. langaa*	Chicken	−
CCUG3713^T	*P. volantium*	Fowl	−
CCUG18782	*P.* sp. A	Chicken	−
NCTC11414	Bisgaard Taxon 2	Duck	−
NCTC11412	Bisgaard Taxon 3	Duck	−
B7/99/1	Bisgaard Taxon 3 biovar 1	Budgerigar	−
S/C1101	Bisgaard Taxon 14 biovar 1	Turkey	−
F75	Bisgaard Taxon 22	Chicken	−
F66	Bisgaard Taxon 26 biovar 1	Duck	−
F97	Bisgaard Taxon 26 biovar 2	Duck	−
HPA106	Bisgaard Taxon 32	Sparrow Hawk	−
101	Bisgaard Taxon 33	Parrot	−
B301529/00/1	Bisgaard Taxon 40	Gull	−
SA4461	*Haemophilus paragallinarum* biovar 2	Chicken	−
4237/2sv	*Riemerella anatipestifer*	Chicken	−
G9	*Salmonella enterica* serotype Gallinarum	Chicken	−
14R525	*Escherichia coli*	Human	−
726-82^T	*Coenonia anatina*	Duck	−
CCUG23171^T	*Ornithobacterium rhinotracheale*	Turkey	−

[a] Superscript T indicates the type strain. [b] + indicates a strong hybridization signal, - indicates no signal.

Figure 1

FISH and histopathology demonstrating the ability of probe GAN850 to hybridize with *Gallibacterium anatis* in pure culture and in infected formalin-fixed tissues. Scale bars indicate the magnification of the images.
(A) Pure culture of *G. anatis* strain 12656-12 yields a bright signal with GAN850.
(B) Pure culture of *Coenonia anatina* hybridizes and gives a clear signal with EUB338.
(C) Splenic tissue from a chicken infected intravenously with *Gallibacterium* showing basophilic aggregates of bacteria in the ellipsoids (open black arrow) surrounding the penicillium capillaries (full black arrow)(HE stain).
(D) Splenic tissue from a chicken infected intravenously with *Gallibacterium* and hybridized with GAN850 yielding a bright signal in patterns corresponding to the basophilic aggregates observed in (C).
(E) Liver tissue from a chicken infected intraperitoneally with *Gallibacterium* and hybridized with GAN850 showing single cells of *G. anatis* positioned on the serosal side of the liver.
(F).
As (E) but hybridized with non-Gan850 showing no hybridization signal.



Discussion

To our knowledge, the FISH procedure developed in the present study is the only method that allows specific identification of *Gallibacterium* in both culture and tissue samples. This is of particular importance as detection and identification of these bacteria remain problematic due to the ambiguity of phenotypical interpretation associated with difficulties at differentiating *Gallibacterium* from other bacterial genera based on phenotypical methods (9). FISH based methods have demonstrated applicability for identification of bacteria in their natural environment (7,20,21). Moreover, a recent study by Tegtmeier *et al*. (29) demonstrated bacterial cultivation to be the least sensitive detection method in tissue in comparison to *in situ* hybridization, immunohistochemistry and PCR, underlining the importance and necessity of alternatives to traditional detection methods. *Gallibacterium* spp. have the typical characteristics of an opportunistic pathogen, relying on predisposing factors, such as co-infections of viral, bacterial or parasitic origin, stress or hormonal imbalances, to elicit disease (13,25). Identification of *Gallibacterium* spp. in mixed bacterial population using a non-culture dependent identification method would be advantageous and avoid selection of certain bacterial species which could distort interpretation of the actual species composition in the sample investigated (8). This is further underlined by the fact that certain isolates of haemolytic *Gallibacterium* only grow under micro-aerophilic conditions. We did not test the method on naturally infected chickens, where profound histological changes and the presence of a mixed bacterial population may lower the performance of the present technique. However, the very bright signal observed from the experimentally infected tissue indicates that no major problems are likely to be expected with regards to the signal intensity from *G. anatis* in naturally infected tissue.

Minor variation in signal intensities was observed between repeated hybridizations with the same strain under identical hybridization conditions, however, the magnitude of variation was negligible and a clear distinction between positive and negative signals was always possible.

Lack of specificity is commonly encountered at development of a genus or species specific probe (20). Sequence alignments to published 16S rRNA sequences in the present study did not identify any homologous matchings. These results were further supported by specificity testing using GAN850 and EUB338 in parallel confirming the accessibility and abundance of 16S rRNA in all the tested non-*Gallibacterium* species included. These did not give signals upon hybridization with GAN850, but clearly detectable signals with EUB338. The GAN850 was also specific when hybridizations were carried out on chicken tissue. We did not observe unspecific binding, cross hybridization or autofluorescence at a magnitude disturbing the signal from *Gallibacterium*, which often appear to hamper visualization of hybridization

signals in tissue (18). The present oligonucleotide labeled with Cy3™ and Cy5™ gave a clear bright signal, which enabled the use of narrow-band-pass filters, enhancing the signal to noise ratio considerably compared to classical fluorophores, such as fluorescein- and rhodamine derivatives, confirming the observations of Wessendorf and Brelje (30). However, some counter staining can be useful to visualize the spatial distribution of bacteria within the tissue (7,17), which in the present study was achieved by the use of a double filter set, allowing passage of signals from the labelling dye as well as specified wavelengths of autofluorescence from the host tissue.

In conclusion, we have designed an oligonucleotide probe targeting 16S rRNA, which enables specific detection *Gallibacterium* species *in situ*. The method represents the first described genotypical diagnostic alternative to the present phenotypically based detection method of *Gallibacterium*. A particular advantage is its ability to identify *Gallibacterium* spp. in infected tissues, which can be of major importance at elucidating pathogenesis and bacteria-host cell interactions of this poorly understood microorganism.

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Chapter 6

In vivo **studies of** ***Gallibacterium anatis*** **infections in chickens**

In press in
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In vivo studies of *Gallibacterium anatis* infection in chickens

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Summary

The aim of the present study was to investigate the pathology in normal or immunosuppressed chickens followed intravenous or intraperitoneal inoculation with a well characterized strain of *Gallibacterium anatis*. Two groups of 30 15-week-old commercial brown laying chickens were used, having been screened and found negative for *Gallibacterium* organisms. One group was treated with 5-fluorouracil to promote heterophil-depletion, while the other was saline-treated. Ten days later 15 chickens from each group were inoculated either intravenously or intraperitoneally with $3.3x10^7$ colony-forming units of *G. anatis* strain 12656-12. Subsets of chickens were sacrificed at 3, 12 or 24 hours post infection and examined for lesions. Livers and spleens were examined by culture and by fluorescent *in situ* hybridization. Intravenously infected birds showed severe septicaemic lesions in both the normal and immunosuppressed birds. Mortality was recorded only in the later, with an over-all rate of 73%. The intraperitoneally infected chickens of normal immune status showed various degrees of localised purulent peritonitis at the inoculation site, but in the immunosuppressed birds the entire peritoneum tended to be involved along with the abdominal organs. This was similar to previous descriptions of natural infections and may represent a useful infection model for detailed analysis of *Gallibacterium* virulence factors and pathogenesis.

Introduction

Gallibacterium was recently established as a new genus within the family *Pasteurellaceae* Pohl 1981 (Christensen *et al*., 2003). Bacteria belonging to this genus have previously been reported as *Pasteurella anatis*, avian *Pasteurella haemolytica*-like organisms or *Actinobacillus salpingitidis*.

The pathogenic potential reported for *Gallibacterium* is highly variable. These organisms have been isolated from clinically healthy birds, in which they have been suggested to constitute a part of the upper respiratory tract and lower genital tract flora (Harry, 1962; Bisgaard, 1977; Mushin *et al*., 1980; Bojesen *et al*., 2003a). However, others have isolated *Gallibacterium* in pure culture from diseased chickens affected with salpingitis, oophoritis, peritonitis, septicaemia, pericarditis, hepatitis and upper respiratory tract lesions, indicating that at least some strains of *Gallibacterium* possess a pathogenic potential (Kjos-Hanssen, 1950; Greenham & Hill, 1962; Harbourne, 1962; Gerlach, 1977; Bisgaard & Dam, 1981; Addo & Mohan, 1984; Shaw *et al*., 1990; Mirle *et al*., 1991; Suzuki *et al*., 1996; Suzuki *et al*., 1997a; Suzuki *et al*., 1997b). Although no specific lesions have been assigned *Gallibacterium* infections, some reports have indicated that these bacteria may be involved in infections of the salpinx and the peritoneum in particular (Kohlert, 1968; Hinz, 1969; Matthes *et al*., 1969; Gerlach, 1977). This view was substantiated by Mirle *et al*. (1991) who, in a post mortem investigation including 496 hens, isolated *Gallibacterium* as one of the most frequent bacterial agents from lesions in the reproductive tract. Previous experimental infections with *Gallibacterium* have shown substantial variation with regard to mortality, ranging from low or non-pathogenic (Harbourne, 1962; Bisgaard, 1977; Mushin *et al*., 1980) to highly pathogenic, with mortality rates reaching 85-90% (Matthes & Löliger, 1976; Gerlach, 1977). However, differences in the experimental set-up as well as uncertainty with regard to correct classification of organisms now classified as genus *Gallibacterium* have placed some doubt upon interpretation of these results (Bisgaard, 1993).

Consequently, the aim of the present study was to provide a basic understanding of bacteria-host interaction through a controlled infection study using a well characterized *Gallibacterium* strain in chickens of different immune status. Immunosuppression was achieved by depleting the heterophil cells, which play a major role in the innate cellular immune response in chickens (Bounous & Stedman. 2000). Two inoculation routes were used. Intravenous (i.v.) inoculation was included to provide basic information on virulence, whereas inoculation by intraperitoneal (i.p.) route was included to mimic peritoneal infection, from which *Gallibacterium* have often been isolated after natural infection (Gerlach, 1977; Bisgaard & Dam, 1981; Mirle *et al*., 1991).

Materials and methods

Experimental animals and housing facilities

Fifteen-week old chickens (Lohmann Brown layers) were obtained from a commercial farm that had a high biosecurity level. A random sample of cloacal swabs from 30 chickens, which allows detection of infection levels greater than 10%, with 95% certainty (Martin *et al*., 1987), was collected for bacterial cultivation on blood agar base (Oxoid, Hampshire, England) with 5% bovine blood (BA) to ensure freedom from *Gallibacterium* infection. Birds were allowed to acclimatise for one week and were provided water and food *ad libitum* and kept together under free-range housing conditions without outdoor access. All work on experimental animals was carried out with the approval of the Danish National Animal Ethics Committee.

Bacterial strains, media and preparation of inoculum

Gallibacterium anatis strain 12656-12 originally isolated from the liver of a chicken with septiceamia. This strain has been characterized in detail by phenotypical and genotypical methods and was therefore found suitable for further studies of pathogenicity (Bojesen *et al*., 2003b). Bacteria were stored at –80 ^{o}C and cultivated over night (ON) on BA. Single colonies were incubated with shaking in Brain Heart Infusion broth (BHI) (Oxoid) at 37 ^{o}C and subsequently stored at 4 ^{o}C, in order to synchronise the population in stationary growth phase. Prior to use, 12 µl of the primary culture of each tube was added to 10 ml BHI following incubation at 37 ^{o}C until the culture reached late log-phase. Bacterial numbers and growth phase were estimated at OD_{600} and adjusted to a concentration of approximately 10^8 cfu/0.5 ml with 1 X phosphate buffered saline (PBS) prior to inoculation. The bacterial concentration in each inoculum was verified by plate counts on BA plates in duplicate.

Immunosuppressive treatment and heterophil counts

5-Fluorouracil (Faulding Pharmaceuticals, Warwickshire, UK), (50 mg/ml, pH 9.0) was given at a dosage of 200 mg/kg body-weight by a single i.v. injection into the jugular vein. The control group received an equal volume of sterile, pyrogen-free saline. Blood samples from each bird were collected at the time of 5-FU/saline treatment, day 0 (D_0) and at the time of inoculation with *G*. *anatis* 12656-12, ten days after 5-FU treatment (D_{10}). Blood samples (0.3-0.4 ml) were collected from each bird with a syringe (27G needle) into Vacutainer™ blood collection tubes containing EDTA (Becton & Dickinson, New Jersey, US). The blood samples were gently inverted five to10 times to ensure mixing with EDTA and kept at 4 ^{o}C for further use. The blood was subsequently diluted 1:20 – 1:40 in an eosin:acetone solution (eosin, 100 mg; sodium citrate hydrate, 300 mg; acetone, 15 g, made up to 100 ml with distilled water) for the heterophil count. The number of heterophil granulocytes was counted in a

Fuchs-Rosenthal haemocytometer. The counts were completed within 48 h of blood collection. All eosin-stained cells were counted and no attempts were made to differentiate heterophils from eosinophilic granulocytes. The average heterophil count for the chickens in each treatment group, at D_0 and D_{10}, was compared using Students t-test for significance of differences between means of paired data.

Experimental infection of heterophil-depleted and non-depleted chickens

Sixty 15-week-old chickens were divided randomly into two groups of 30 and on experimental day 0 (D_0), one group was treated with 5-FU and the other with saline. Ten days later (D_{10}), each treatment group was randomly divided into two further of 15. One subgroup of 5-FU treated birds and one of saline-treated birds were inoculated i.v. with *G. anatis* while the other two subgroups were inoculated i.p.

Five birds from each of these subgroups were then selected randomly and killed by decapitation 3 (D_{10+3h}), 12 (D_{10+12h}) or 24 (D_{10+24h}) hours after bacterial inoculation, in order to evaluate the pathological lesions developing over time. All were subjected to *post mortem* examination. Bacterial counts from spleen and liver samples were obtained by streaking tissue swabs onto plates of blood agar base (BA), (Oxoid, Ballerup, Denmark) supplemented with 5% citrated bovine blood. Isolates were identified as *Gallibacterium* if they exhibited greyish, semitransparent colonies surrounded by a wide *β*-haemolytic zone (1-2 mm), had a butyrous consistence, were smooth, shiny and circular and raised with an entire margin and a size of 1-2 mm in diameter after 24 h at 37 ^{o}C and meeting the genus criteria outlined by Christensen *et al*. (2003).

Histopathology and 16S rRNA in situ hybridization

Samples of liver and spleen were kept in 10% phosphate-buffered formalin for up to 24 hours followed by dehydration, embedding in paraffin wax and preparation of 3 to 4 μm thick cross sections. Sections were mounted on adhesive slides (Super Frost/Plus, Menzel-Gläser, Germany) and stained with haematoxylin and eosin (HE) stain.

Fluorescent *in situ* hybridization (FISH) on liver and splenic tissue was performed with a *Gallibacterium* specific probe GAN850 as reported previously (Bojesen *et al*., in press)

Results

Heterophil counts

The average heterophil count at D_0, in the 5-FU treated chickens, was 3950 (SD±1430) cells/mm^3 compared to 90 (SD±5) cells/mm^3 at D_{10}, corresponding to a 44 fold and highly significant reduction ($P<0.001$). There was no significant reduction ($P>0.25$) in the average heterophil counts from D_0, (4030 (SD±1070) cells/mm^3) to D_{10}, (3910 (SD±970) cells/mm^3), in the saline treated chickens.

Intravenously infected chickens

The results from the post mortem examinations are summarized in Table 1. The infection induced severe depression of the chickens, which were reluctant to move. In addition, a number of birds developed acutely inflamed bursa presternalis, probably as a consequence of unnaturally long periods of resting on their breasts. Both the 5-FU treated and untreated chickens, showed severe vascular lesions as a consequence of the induced septicaemia. Red discolouration of fat and extensive serohaemorrhagic exudation into all body cavities was apparent as early as 3 h p.i. Histopathology of the spleens demonstrated non-identifiable necrotic cells, proteinaceous fluid, eosinophilic and basophilic aggregates and in the ellipsoids (Fig. 1A). The basophilic aggregates were shown to be microcolonies of *Gallibacterium* by the positive signal obtained with FISH (Fig. 1B). The liver lesions were less apparent and included basophilic aggregates (Gallibacterium microcolonies) bordered by necrotic hepatocytes in the treated birds.

A number of chickens from the treated group had to be killed before the scheduled sampling at 12 h p.i., due to their very poor condition, but both the 5-FU-treated and saline-treated chickens exhibited excessive vascular changes and multiple necrotic foci in the spleens and livers. However, the gross pathological changes were more profound in the 5-FU-treated compared to the saline-treated chickens. The splenic lesions in the saline-treated birds were similar to the ones showed in figure 1A. In the 5-FU-treated birds, the splenic lesions had larger necrotic areas involving the peri-arterial-lymphatic-sheath (PALS), as well as pronounced oedema in the organ. The histological changes in the livers were less apparent than the splenic lesions, although bacterial colonies bordered by necrotic hepatocytes were found in the 5-FU-treated chickens.

At 24 h p.i. the macroscopic changes were mostly similar to those observed after 12 h p.i. including vascular changes and multiple necrotic foci. The histopathological changes were also similar to the ones observed at 12 h p.i. However, the formation of multinucleated giant cells was apparent around some of the ellipsoid lesions in the spleen.

Figure 1

1A) Spleen from an intravenously inoculated and heterophil-depleted chicken, 12 h post inoculation. Splenic penicilium arteriole (full arrow) surrounded by ellipsoids filled with degranulated heterophils, eosinophilic aggregates and proteinaceus fluid (open arrow)(HE stain).
1B) Spleen section (as 1A) demonstrating a signal distribution corresponding to the eosinophilic aggregates observed in 1A showing microcolonies of *Gallibacterium* (FISH).
1C) Liver from an intraperitoneally inoculated and heterophil-depleted chicken, 12 h post inoculation. Blood filled sinousoids and a thick perihepatic layer of pus (arrow) including *Gallibacterium* and erythrocytes (FISH).
1D) Liver from a intraperitoneally inoculated and heterophil-depleted chicken, 12 h post inoculation. Single cells (open arrow) and microcolonies (full arrow) of *Gallibacterium* visualized intra- and subcapsularly (FISH).

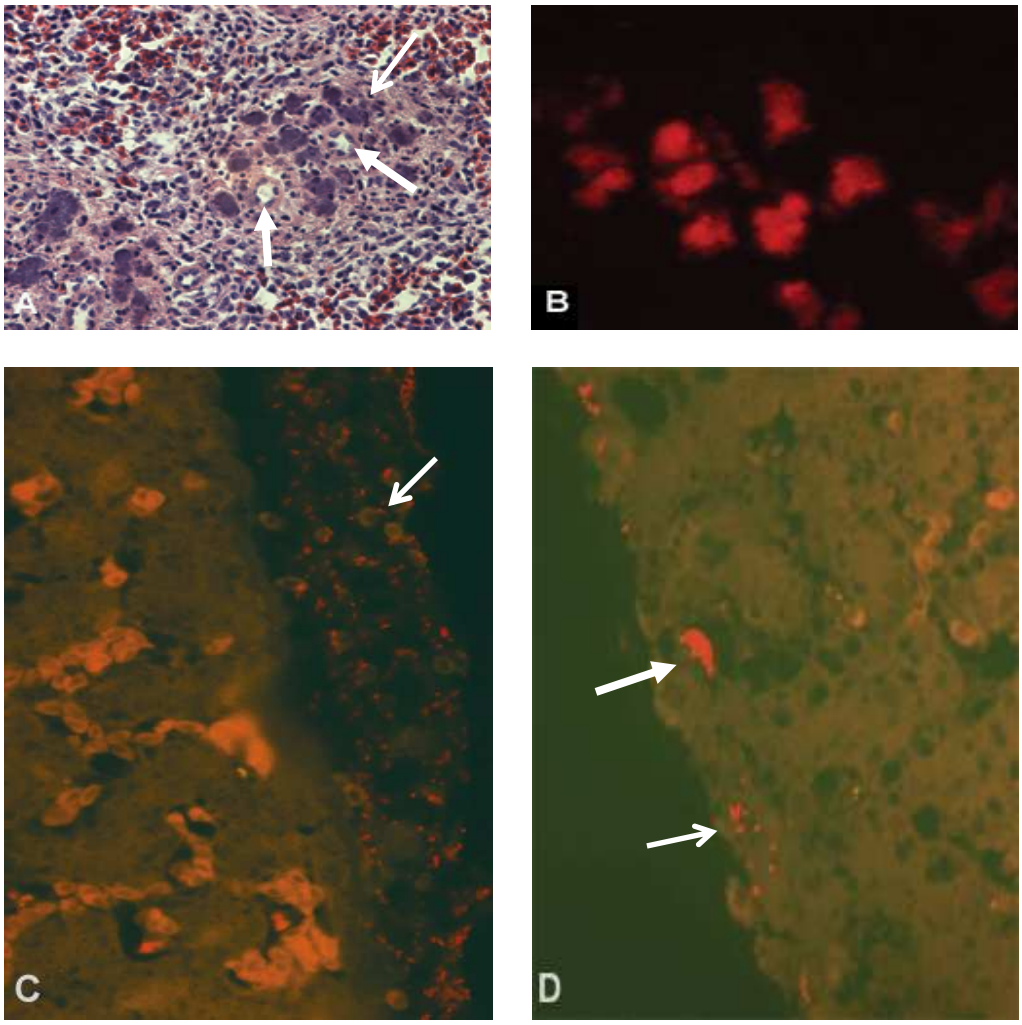

Table 1. *Number of culture positive animals, mortality and lesions at different times post i.v. inoculation of Gallibacterium anatis in chickens*

Hours (p.i.)	Treatment	Group size	*Gallibacterium* culture positive		Macroscopic lesions (no. of birds)
			Liver	Spleen	
3	5-FU	5	4	4	Mortality (2). Severe, red discoloration of fat and serous membranes (2). Severe (2) or mild (3) serohaemorrhaic exudation to the peritoneal cavity. Ecchymotic (2) or petechial (3) haemorrhages in the myocardium and the cardiac fat. Moderate splenomegaly (2).
	Saline	5	5	5	Petechial haemorrhages in the myocardium and coronary fat (5). Moderate splenomegaly (5). Mild exudation to serous membranes (5). Focal red discoloration of abdominal fat (5).
12	5-FU	8[a]	8	8	Mortality (8). Hepatomegaly and splenomegaly (8). Pronounced serohaemorrhagic exudation on all serous membranes (8). Extensive haemorrhages on the serosal surface of the proventriculus (8). Markedly diffuse necrosis of liver and spleen including texture changes (8). Acute bursitis presternalis (4).
	Saline	5	4	4	Extensive serohaemorrhagic exudation from all serous membranes (5). Hepatomegaly and splenomegaly (5). Petechial haemorrhages in the myocardium and cardiac fat (5). Multifocal necrosis of liver and spleen, texture close to normal (5). Acute hemorrhagic bursitis presternalis (3).
24	5-FU	2	2	2	Mortality (1). Diffuse, red discoloration and pronounced serohaemorrhagic exudation from serous membranes (1). Hepatomegaly and splenomegaly (2). Petechial haemorrhages in the myocardium and the cardiac fat (1). Pale coloured liver with necrosis and texture changes (2). Multifocal necroses in spleen (1).
	Saline	5	5	5	Moist serous membranes (5). Petechial haemorrhages in the coronary fat (5).Pale liver with changes in texture (5). Multifocal pin-point necrosis in spleen and pronounced splenomegaly (5).

[a]Only five birds should have been killed at this time but eight were severely affected and were killed at 5-6 h p.i.

Table 1 (-continued). *Number of culture positive animals, mortality and lesions at different times post i.p. inoculation of Gallibacterium anatis in chickens*

Hours (p.i.)	Treatment	Group size	*Gallibacterium* culture positive		Macroscopic lesions (no. of birds)
			Liver	Spleen	
3	5-FU	5	0	2	Haemorrhages at the site of injection.
	Saline	5	0	0	Haemorrhages at the site of injection.
12	5-FU	5	3[b]	2[b]	Diffuse fibrineous perihepatitis (4). Purulent peritonitis with localisation ventrally corresponding to the inoculation site, but with a tendency to involve to the entire peritoneum (4). No macroscopic lesions (1).
	Saline	5	0	0	Local purulent peritonitis at the site of inoculation (4). No macroscopic lesions (1).
24	5-FU	5	0	0	Diffuse purulent peritonitis (1). Local haemorrhages in the peritoneal fat at the site of injection (2). No macroscopic lesions (2).
	Saline	5	0	0	Splenomegaly with multifocal greyish pint-point foci (5). Diffuse perihepatitis (1).

[b]In total 4/5 birds were culture positive in the spleen, the liver or in both organs.

Intraperitoneally infected chickens

The severity of the gross and microscopic lesions differed markedly between the 5-FU-treated and the saline-treated group, which was underlined by the difference in mortality between the groups, where 11 out of 15 (73%) died in the 5-FU-treated group, compared to 0/15 (0%) among the saline-treated chickens.The i.p. inoculated chickens showed only minor macroscopic lesions at 3 h p.i. However, bacteria had already gained access to the lymphatic system in two of the 5-FU treated birds, as shown by positive cultures obtained from the spleens. No histopathological lesions were observed at this time in either spleen or liver tissue from any of the birds.

By 12 h p.i., a diffuse purulent peritonitis and fibrineous perihepatitis was apparent in 4/5 of the 5-FU treated birds, some of which showed signs of systemic infection by positive bacterial culture from both livers and spleens. However, no histological reaction was observed in those organs in any of the birds examined. One bird was culturally negative in the liver and spleen, however it was possible to identify basophilic aggregates by HE stain which later were confirmed to be *Gallibacterium anatis*. The only histological changes observed in the spleen of this bird were thrombosis in some of the central veins.

Generally, purulent masses containing bacteria and erythrocytes were demonstrated by FISH on the serosal surface of the livers of the 5-FU-treated birds (Fig. 1C). Additionally, microcolonies and single cells of *Gallibacterium* were demonstrated intra- and subcapsularly in the livers by FISH (Fig. 1D).

At 24 h p.i. none of the birds cultured positive from the liver or spleen and only one of the 5-FU treated chickens had diffuse purulent peritonitis. No histopathological changes were observed in the livers or spleens from either treated or untreated birds.

Discussion

Although avian heterophils have been shown to generate relatively low amounts of oxygen free-radicals and have less phagocytic capability than human and canine neutrophils, their bactericidal abilities seems to be at a similar level (Kogut *et al*., 1993; Brooks *et al*., 1996). The anti-cancer drug, 5-fluorouracil (5-FU), has been used extensively to generate neutropenic mice as models for the study of human bone marrow transplantation and cancer treatment (Ardalan & Glazer, 1981; Donowitz & Quesenberry, 1986). In the present study, 5-FU treatment resulted in an approximately 44 fold reduction of circulating heterophils, confirming previous observations in chickens (Kogut *et al*., 1993; Raj *et al*., 1997). Chickens do not possess a reservoir of preformed granulocytes extravascularly (Glick & Rosse, 1976; Toth & Siegel, 1986) and as this cell line has a high turn-over rate and is dependent on a continuous in-flow of newly formed cells from the bone marrow, a single administration of 5-FU promotes a reversible reduction of the granulocytic cells in the chicken. We did not investigate whether blood cells other than granulocytic cells were affected by the cytotoxic effects of 5-FU and our cell staining method did not differentiate between subgroups of granulocytic cells. However, previous studies including differential counts have shown that 5-FU primarily acts on the granulocytic precursors in the bone marrow of chickens (Kogut *et al*., 1993). In addition, adverse effects on eosinophils and basophils are unlikely to be of significance in the present study, since the numbers of these cells are low in the normal chicken (Bounous & Stedman, 2000).

The onset of pathological lesions and mortality happened very quickly in the present study, resulting in a high number of severely affected individuals within 5-6 hours p.i. We have previously observed a similar response in a preliminary study using 50 weeks old layers, inoculated i.v. with the same dose and strain of *G. anatis*, where seven out of nine chickens succumbed within 4 hours p.i. with lesions very similar to the ones described for the i.v. inoculated birds in the present work (A.M. Bojesen and M. Bisgaard, unpublished results). The chickens from the preliminary study were obtained from a commercial flock still in

production, and were healthy according to clinical observations. However, we did not attempt to define their immune status specifically, and it may have been partly impaired, explaining the high mortality rate and the severe pathological lesions. The results from the preliminary and present studies indicate that at least certain strains of *Gallibacterium* may play a role as a pathogen in apparently healthy chickens. This contradicts some earlier investigations which suggested that only severely debilitated chickens are likely to be affected by a *Gallibacterium* infection (Bisgaard, 1977; Gerlach, 1977).

The *G. anatis* strain 12656-12 used in the present study was originally isolated from a hen dying from septicaemia in a commercial flock. Another isolate from a hen in the same flock with similar lesions was subjected to genomic fingerprinting, which revealed that the two isolates represented identical clonal types, indicating that this strain could be particularly virulent (Bojesen *et al*., 2003b). This may also explain the serious lesions in the birds from the present study compared to previous studies which may have involved less virulent strains. The basis for differences in pathogenic potential is not known, but is likely to be governed by the possession and expression of virulence factors. No investigations have, however, to our knowledge, been performed to characterise specific factors that could be of significance to *G*. *anatis.* Some apparent candidates do exist as most clinical isolates of *G*. *anatis* from chickens are strongly haemolytic when grown on media containing bovine, horse, rabbit or chicken blood (Greenham & Hill, 1962; Christensen *et al*., 2003). Closely related bacteria also encode haemolytic toxins and those of the RTX toxin family seem to be especially widespread among members of the family *Pasteurellaceae*, as the toxins LktA in *Mannheimia haemolytica* (Lo *et al*., 1989) and ApxI-IV in *Actinobacillus pleuropneumoniae* (Chang *et al*., 1989; Frey *et al*., 1991; Chang *et al*., 1993; Schaller *et al*., 1999) have been shown to be important for virulence. A similar role may be ascribed to Aqx in *Actinobacillus equuli*, but this remains to be shown *in vivo* (Kuhnert *et al*., 2000). Certain strains of *Gallibacterium* also produce a capsule, which may be of importance as a virulence factor as has been shown for *Pasteurella multocida* (Boyce & Adler, 2000). The roles of these putative virulence factors remain, however, pure speculation and a subject for further analysis.

Several host and environmental factors may also influence the outcome of a *Gallibacterium* infection. Hormonal imbalances (Gerlach, 1977), co-infections with other microorganisms (Shaw *et al*., 1990) and “cold stress” (Matthes & Löliger, 1976) have all been indicated as being of importance. Matthes and Löliger (1976) inoculated *Gallibacterium* into the crop of normal and “cold stressed” chickens, which after 48 hours showed a mortality rate of 0% (0/20) and 36% (9/25), respectively. Their results indicated that even though *Gallibacterium* may be a harmless inhabitant under normal conditions, adverse effects may

be initiated by various stressors permitting invasion and even mortality as seen in heterophil depleted chickens in the present study.

Although no specific syndrome has been associated *Gallibacterium* infection, a number of reports have indicated salpingitis and peritonitis as the dominant lesions (Hinz. 1969; Gerlach, 1977; Bisgaard & Dam, 1981; Mirle *et al*., 1991). We inoculated the chickens with *Gallibacterium* intraperitoneally in order to mimic a peritoneal infection following an ascending bacterial infection through the oviduct. All chickens developed some degree of purulent peritonitis within 12 hours p.i., which in the 5-FU treated birds developed to involve the entire peritoneum. Furthermore, there was evidence of bacterial invasion of the spleen and liver as shown by positive culture and by FISH. These results indicate that at least *G. anatis* strain 12656-12 has the ability to promote peritonitis and gain systemic access corresponding to naturally occurring cases of *Gallibacterium* infections. This is of importance as this is the first controlled study of *Gallibacterium* infection where both the inoculated strain and the immune status of the host were defined in detail. Our results may therefore have important implications for future work attempting to elucidate the nature of the virulence factors discussed above where intraperitoneal infection of 5-FU treated chickens may act as an infection model for this purpose.

In one of the chickens inoculated i.p., *Gallibacterium* was identified in the liver by FISH although bacterial cultivation was negative. This indicated that FISH may be more sensitive for identifying *Gallibacterium* in infected tissue than the traditional methods based on bacterial cultivation. Similar results were demonstrated for the identification of *Haemophilus somnus* from naturally infected bovine lungs (Tegtmeier et al., 2000), which emphasises the need of alternative detection methods to complement the culture based at establishing a definitive diagnosis.

In conclusion, we confirmed that 5-FU is a potent drug for mediating heterophil-depletion in chickens in order to study the pathogenesis of bacteria with a low- to moderate virulence. We showed that i.v. and, in particular, i.p. infections resulted in pathological lesions comparable to what has been reported previously from naturally infected chickens, indicating that the present protocol may act as a model for future studies of the *G. anatis* pathogenesis.

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Chapter 7

Cloning and characterization of the gene encoding *Gallibacterium anatis* FnrG, a regulator of the *Escherichia coli* silent haemolysin SheA

Preliminary work

Cloning and characterization of the gene encoding *Gallibacterium anatis* FnrG, a regulator of the *Escherichia coli* silent haemolysin SheA

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Abstract

Fnr is a global regulator in *Escherichia coli* involved in the regulation of a number of genes during the transition to and under anaerobic growth. The *fnrG* gene homologue from *Gallibacterium anatis*, which confers a haemolytic phenotype in *Escherichia coli,* was partly sequenced and its role in the expression of the *Escherichia coli* silent haemolysin, *sheA* was investigated.

The predicted amino acid sequence of FnrG showed 85% (182 out of 214 amino acids) identity to the *Actinobacillus actinomycetemcomitans* Fnr protein and 84% (180 out of 214 amino acids) identity to *Pasteurella multocida* Fnr. A 24 amino acid conserved region in the C-terminal end, encoding a putative DNA binding domain, was identified. Furthermore, a sequence encoding a highly conserved cysteine residue in position 122, likely to be involved in metal ion binding and O_2 sensing and essential for Fnr activity, was also demonstrated.

The activity of FnrG was investigated by transforming a 4.2 kbp *Hin*d fragment containing *fnrG* into an *E. coli* Δ*sheA*, a null mutant of the silent haemolysin. The resulting transformant was non-haemolytic, which indicates that FnrG activates *sheA* and is likely to be a member of the Fnr global regulatory protein family.

Introduction

Gallibacterium have recently been established as a new genus in the family *Pasteurellaceae* Pohl 1981. The genus encompasses one species, *G. anatis*, and two additional genomospecies (Christensen *et al*., 2003).

Gallibacterium have been isolated from both healthy (Mráz *et al*., 1976; Bisgaard, 1977; Bojesen *et al*., in press) and diseased poultry (Kjos-Hanssen, 1950; Harbourne, 1962; Gerlach, 1977). Salpingitis with or without peritonitis has in particular been associated with *Gallibacterium* infections, although no pathognomonic lesions is produced (Kohlert, 1968; Mirle *et al*., 1991).

Little is known about factors governing the virulence of *Gallibacterium*, however, many strains of *Gallibacterium* have a haemolytic phenotype. Greenham and Hill (1962) made the initial characterization of the *Gallibacterium* haemolysin and demonstrated its haemolytic activity on equine, bovine, rabbit and chicken blood agar. After 24 hours *Gallibacterium* forms a grayish, semitransparent colony surrounded by a wide *β*-haemolytic zone (1-2 mm). Haemolysins/cytotoxins have in closely related species been shown to be important for virulence. The leucotoxin in *Mannheimia haemolytica* (Lo *et al*., 1987), and ApxI, ApxII and ApxIV in *Actinobacillus pleuropneumoniae* (Chang *et al*., 1989; Frey *et al*., 1991; Schaller *et al*., 1999) are all of the RTX type (Schaller *et al*., 2000; Narayanan *et al*., 2002) and have been shown to be virulence factors in the pathogenesis of “shipping fever” (Stevens and Czuprynski, 1996) and porcine pleuropneumoniae (Chang *et al*., 1993; Jansen *et al.,* 1995), respectively.

The aim of the present study was to clone and characterize the genetic elements responsible for the haemolytic phenotype of *Gallibacterium anatis*.

Bacterial strains, growth conditions and recombinant DNA techniques. Strains and plasmids used in this study are described in Table 1. *Gallibacterium anatis* was grown in Brain Heart Infusion (BHI) broth (Difco laboratories, Detroit, Mich.) or on BHI agar plates supplemented with 5% citrated bovine blood. *E*. *coli* were cultured in 2YT (Oxoid, Hampshire, England) broth or on 2YT agar plates, supplemented with 5% bovine blood for selection of haemolytic strains. Antibiotics were added when required in concentrations: kanamycin (50 µg/ml) and ampicillin (100 µg/ml). *G. anatis* and *E. coli* genomic DNA were isolated by the hexadecyltrimethyl ammonium bromide (CTAB) method as described previously (Ausubel *et al*., 1987). However, *G. anatis* was pretreated with lysozyme at 37 ^{o}C for 24 hours. Plasmid DNA was prepared by alkaline lysis (Birnboim and Doly, 1979) or by Qiagen (Hilden, Germany) anion-exchange colums. DNA restriction was performed with enzymes obtained from Roche Molecular Biochemicals (Basel, Switzerland) or New England Biolabs (Beverly,

Mass.), and carried out according to the manufacturers' instructions. DNA was introduced into *E. coli* by chemical transformation as previously described (Ausubel *et al*., 1987). DNA sequencing was carried out using the BigDye Ready Reaction DyeDeoxy Terminator cycle sequencing kits (Perkin-Elmer, Foster City, Calif.) and analyzed with a ABI 373 automated sequencer (PE Biosystems). PCR amplification was performed using Taq polymerase essentially as described by Saiki *et al*. (1988).

Table 1. Bacterial strains and plasmids

Strain	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	F$^-$ *endAI hsdR17* (r_k^-, m_k^+) *thi-l* λ$^-$ recA1 gyrA96 relA1 ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories, MD, USA
MC4100	F$^-$ *araDI139 DlacU169 rspl150 relA1 thiA flbB5301 deoC1 ptsF25 rbsR*	del Castillo *et al*., 1997
CFP201	MC4100 *sheA*::Tn5-2.1 null mutant	del Castillo *et al*., 1997
SBA447	DH5α harboring pWSK29	K. Rajakumar[a]
Gallibacterium anatis		
12656	Clinical isolate from the liver of a chicken that died with septiceamia.	Bojesen *et al*. (2003)
Plasmids		
pWSK29	Amp^r, *lacZ'*, pSC101 origin, f1 origin, low copy plasmid	Wang and Kushner, 1991
pAMB1	pWSK29::4.2-kb *Hin*DIII *G. anatis* fragment, *fnr*$^+$	This work

[a]Constructed by Kumar Rajakumar at Monash University, Department of Microbiology, Victoria, Australia.

Identification of a haemolytic clone and determination of the nucleotide sequence of *fnrG.* A recombinant DNA library of *G. anatis* genomic DNA was constructed by ligating *Hin*dIII fragments into the plasmid pWSK29. The *E. coli* DH5α strain was transformed to kanamycin resistance with ligated DNA, and the resulting colonies were screened for haemolysis on 2YT agar containing 5% citrated bovine blood. Haemolytic colonies were surrounded by zones of clear lysis for of up to 0.5 mm in width. A plasmid pAMB1 containing a 4.2 kb insert was isolated from two haemolytic colonies. Both inserts was sequenced from both ends, however the sequence of the entire insert was not achieved. Sequence analysis was performed by Kodon 1.0 (Applied maths, Austin, Tx.). Homology search and identification of conserved domains were performed with BlastX (NCBI) and Pfam (Sanger Institute).

Four open reading frames (ORF) were identified. A 669-bp ORF exhibited a high degree of homology to *Actinobacillus actinomycetemcomitans hly-X*-like gene, *Pasteurella multocida*

and *Haemophilus influenzae fnr*, *Actinobacillus pleuropneumoniae hlyX*, and *Mannheimia haemolytica fnrP*. The ORF was named *fnrG* and its translated product displayed 85% (182 out of 214 aa) identity to the *A. actinomycetemcomitans* derived fumarate and nitrite reduction (Fnr) protein and 84% (180 out of 214 aa) identity to the *P. multocida* Fnr.

A second 717-bp ORF upstream of *fnrG* showed 56% identity to an ORF coding for a putative universal stress protein (PMO669) from *P. multocida.* Two ORFs were identified downstream og *fnrG*. The first showed 53% identity to a putative short-chain alcohol dehydrogenase, *fabG*, in *Shewanella oenidensis*, while the second ORF showed 66% identity to *rsgA* from *H. influenzae*, which is predicted to encode a putative non-heme iron storage protein.

fnr is a member of the *fnr*/*crp*-like regulatory gene family (Spiro, 1994). Fnr is a global regulator, which often acts in concert with other global regulators to control the synthesis of a large number of genes during anaerobic conditions (Lynch and Lin, 1996).

Both ends of Fnr have conserved residues; the C-terminal region encodes a helix-turn-helix motif involved in DNA binding, whereas the N-terminal region contains a cluster of cysteine residues (Cys20, 23 and 29) important for Fnr function. A fourth conserved cysteine residue in position 122 was found essential for function (Spiro and Guest, 1988; Melville and Gunsalus, 1990; Sharrocks *et al*., 1990).

The amino acid sequence of the translated product of *fnrG* demonstrated a highly conserved region of 24 amino acids, corresponding to the helix-turn-helix motif described above. Cys122 was also identified in FnrG, but the remaining C-terminal cysteine residues could not be identified as the sequence was terminated at position 44.

The considerable sequence identity between FnrG and various homologues of Fnr, the presence of the conserved Cys122 and the region involved in DNA binding indicates that FnrG is a Fnr-like transcriptional regulator.

A *sheA* mutant transformed with *fnrG* is non-haemolytic. Fnr-like regulatory proteins have been shown to confer a haemolytic phenotype in *E. coli* DH5α and have therefore been indicated to be putative haemolysins or involved in the regulation of haemolysin expression (MacInnes *et al*., 1990; Uhlich *et al*., 1999; Kokeguchi *et al*., 2000; Uhlich *et al*., 2000). *HlyX* from *A. pleuropneumoniae* was found capable inducing haemolysis in *E. coli*, via activation of the silent haemolysin *sheA* (MacInnes *et al*., 1990; del Castillo *et al*., 1997; Green and Baldwin, 1997). FnrP has also been shown to suppress and activate the transcription of *M. haemolytica* leucotoxin under aerobic and anaerobic conditions, respectively (Uhlich *et al*., 2000).

We performed phenotypic control of *fnrG* by transforming pAMB1 into *E. coli* CFP201, a *sheA* mutant (del Castillo *et al*., 1997). The CFP201 transformed with pAMB1 remained non-haemolytic on BA plates. The parent strain, MC4100, lacking the mutation in *sheA*, was also transformed with pAMB1 and showed a haemolytic phenotype corresponding to the phenotype shown with the cloning host, *E. coli* DH5α.

We did not obtain the full length sequence of *fnrG* and were therefore unable to demonstrate the sequence encoding the remaining three C-terminal cysteine residues involved in metal ion binding and oxygen sensing. However in spite of that, we find it most likely that FnrG, based on the identification of the Cys122; the highly conserved N-terminal 24 amino acids sequence and the resulting phenotype after transforming *E. coli* CFP201 with pAMB1, is a homologue to the Fnr global regulatory protein family.

Subsequent studies including sequencing of the entire *fnrG* gene and additional functional studies with the *fnrG* gene alone will be needed to attain more comprehensive characterization FnrG. Furthermore, new investigations will have to be addressed the haemolytic elements responsible for the haemolytic phenotype in *Gallibacterium* as initially attempted in this study.

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Chapter 8

General discussion, future perspectives and conclusions

The work outlined in the present thesis represents two basically different approaches. One approach strives to gain information representing an entire bacterial population (chapters 2-5), whereas the other merely includes the study of the characteristics of a single bacterial isolate (chapter 6-7).

The study of bacterial populations by the use of molecular tools is a fairly young branch of microbiological science, however, in spite of that this field has evolved rapidly during the last two decades (Musser, 1996).

A central parameter when defining and characterizing a bacterial population is the ground material provided for the detailed investigations. Here, a collection of strains, ideally representing the full diversity of a particular bacterial population is an important, but never the less often impossible criteria to meet (Christensen *et al*., 2001). This was exemplified by Frederiksen and co-workers, who in 1999 noted that the number of new named bacterial species and genera, based on a single isolate, had increased rapidly during the preceding decade. This development has created concerns about the reliability of much of the recent taxonomy, as it has been judged unlikely that a single isolate will be able to truly represent the nature of an entire genus or species (Christensen *et al*., 2001).

Consequently, the work described in the present thesis (chapter 2-5) aimed at meeting the minimal number of strains suggested in recommendation 30b of the *International Code of Nomenclature of Bacteria* (Lapage *et al*., 1992).

The present proposal of genus *Gallibacterium* and species included (chapter 2), and the study of the genetic diversity of *Gallibacterium anatis* within defined natural populations by AFLP fingerprinting (chapter 4) was based on more than 30 isolates, carefully chosen from a main collection including a total of more than 350 *Gallibacterium* isolates, in order to account for as mush diversity as possible. Despite attempts to include heterogeneity, our strain collection had an overweight of lesion-associated isolates, a typical bias, as most isolates in strain collections coming from diseased humans or animals due to the natural focus on these areas (Spratt and Maiden, 1999). Whether this had lead to an over-representation of particularly virulent isolates and a neglection of less pathogenic isolates, therefore could be speculated. A biased strain collection narrows interpretational frame and may not allow for conclusions on behalf of the entire population, independently of the number and quality of the analytical approaches applied. This may be of particular importance for the study of a bacterial population like *Gallibacterium*, where the majority of the population probably resides as commensals in healthy birds (Bisgaard, 1977; chapter 2). However, judged on an over-all basis, including the considerable variation in time and space of isolation, the phenotypic divergence of the bacterial isolates and host animal sources characterizing the strain

collection used in our work, we assume that the results and conclusions described in chapters 2-5 can withstand major revisions of the taxonomic definitions.

The establishment of genus *Gallibacterium* along with the tools enabling specific identification and typing will hopefully lead to an increased interest and activity in obtaining information about these bacteria. This may furthermore add to an even broader representation of isolates from different sources, enabling further insight into population structure of *Gallibacterium* and molecular epidemiological studies. Especially studies aiming at elucidating the factors governing host-adaptation would be interesting, as it seems as if different *Gallibacterium* subpopulations have adapted to fairly different ecological niches. In addition to several different bird species from which *Gallibacterium* has been isolated, 10-15 isolates have also been acquired from healthy as well as diseased cattle (Mushin, 1980; Bisgaard, 1993; Bisgaard, unpublished results; Chapter 2). It is not yet known if a host preference of certain clonal lineages exists and if so, if they can exchange freely between different hosts. In the case of *Salmonella* it is well documented that different serotypes of *Salmonella enterica* have adapted to certain hosts (Maynard Smith, 1996). *Salmonella* Derby furthermore seems to have diverged into two different subtypes, one of which colonizes primarily avian host, whereas the other subtype is mainly found in mammalian hosts (Beltran *et al*., 1988). The disease potential of *Gallibacterium* in different host is not well described. Different strains have been recovered from lesions in different bird species, however, a quantitative study across species with the same strain have not been performed as for *P. multocida*, which have been shown to differ significantly in virulence depending on the host (Petersen *et al*., 2001)

Taking evolution of clonal lineages adapted to specific niches one step further, we demonstrated the presence of two clonal lineages of *Gallibacterium* in the same chicken, one primarily occupying the upper respiratory tract whereas the other was primarily isolated from the cloaca (Chapter 4). Similar indications of the presence of site-adapted clones were made from the results of a longitudinal study of avian *E. coli,* where it was shown that different serotypes could be recovered persistently from the trachea or the cloaca of the same bird, over a 26 weeks sampling period (Christensen *et al*., 2000). There may be several important implications of the indicated niche adaptation and existence of different *Gallibacterium* populations within close proximity. The existence of distinct clonal lines in specific anatomical sites of the same bird may indicate that this particular clone possesses specific factors i.e. adhesins, that allows it to colonize this particular place. From a pathogenesis perspective it could be interesting to investigate whether certain clonal lineages of *Gallibacterium* are involved in salpingitis and peritonitis due to these factors.

In addition, insight into host species adaptation of different *Gallibacterium* populations may reveal information about the likelihood of transmission of bacteria between different types of poultry. At present it remains unclear from where chickens in the industrialized production acquire a *Gallibacterium* infection. We demonstrated that vertical transmission is unlikely (Chapter 3), suggesting horizontal transmission as the main mode of transmission. However, a reservoir of *Gallibacterium* is yet to be identified. Survival and transmission of *Gallibacterium* in poultry houses, which are thoroughly cleaned and disinfected between successive flocks seems unlikely for a relatively fragile bacteria like *Gallibacterium*. This view is substantiated by the results from a study of *P. multocida*, demonstrating that four different outbreaks of fowl cholera on the same farm were caused by four different clones, indicating that elimination of the outbreak clone from the farm was possible and that the outbreak clones must have been newly introduced in each case (Muhairwa *et al*., 2000). Consequently, direct or indirect transmission of *Gallibacterium* from free living birds or other animals hosting the bacteria is probable the most likely scenario. Previous studies have indicated that a *Pasteurella multocida* clone isolated from migrating birds in Denmark could be to isolated in a back-yard poultry flock where it caused an outbreak of fowl cholera, emphasizing that transmission by this route may be possible (Christensen *et al*., 1998). Application of the techniques described in chapter 4 and 5 of the present thesis will enable specific identification and genetic typing of *Gallibacterium* isolates that might be recovered from different hosts in the future and may provide valuable information regarding modes of transmission and possible sources of *Gallibacterium.*

In opposition to the population based approach described above, chapters 6 and 7 focuses on an individual isolate in order to characterize issues related to pathogenesis including host and bacterial associated factors. Infection studies in the natural host and detailed analysis of virulence determinants are both types of studies which are bound to be limited to a relatively small number of strains. Infection studies, due to an otherwise unethical use of experimental animals and the detailed characterization of virulence determinants, due to the comprehensive and resource demanding nature of this type work.

Our choice of a particular strain for further characterization was based upon information from the AFLP typing study (chapter 4) that enabled identification of a clone, which may be particularly virulent. *Gallibacterium anatis* strain 12656-12 was initially isolated from the liver of a hen succumbing from septicemia. An identical clone, strain 12656-13, was isolated from the liver of a different hen in the same flock dying from similar lesions. Based on these results we found this clone might represent a worthy candidate for the further infection trials outlined in chapter 6. We were able to reproduce peritoneal lesions in the

immunosuppressed hens, corresponding to what has been reported from previous studies of naturally infected chickens (Gerlach, 1976; Mirle *et al*., 1991). The results seemed to confirm previous reports stating that *Gallibacterium* is a secondary pathogen capable of promoting morbidity and even mortality in the presence of different predisposing factors (Kohlert, 1968, Hinz, 1969).

Given the results from chapter 3, demonstrating a high prevalence of *Gallibacterium* isolates among healthy hens from most Danish productions systems, combined with the results from our infection trials, it may seem surprising that *Gallibacterium* only rarely are reported from naturally occurring cases in Denmark (H.C. Hansen, personal communication) and it could be speculated that *Gallibacterium* as a cause of disease is underestimated. Different reasons may explain why *Gallibacterium* is diagnosed in so few cases. Firstly, despite a relatively high frequency of salpingitis/peritonitis in layers, only a small number of chickens with lesions are actually subjected to a standard bacteriological examination. Secondly, cases that actually are examined bacteriologically may not be fresh, which may have altered the microbial flora within the lesions and suppressed or eliminated *Gallibacterium* if ever present. Thirdly, as indicated in chapter 5 and from studies of *H. somnus* (Tegtmeier *et al*., 2000), standard bacteriological cultivation from lesions may not result in isolation of *Gallibacterium*, in spite of its presence, due to a limited sensitivity of this method. Finally, pathological material obtained from the organic production may be underrepresented, which too may bias the general idea of the importance of *Gallibacterium* in this production. Taken together, future studies addressing issues including survival of *Gallibacterium* in poultry carcasses, evaluation of the lower detection limit with traditional and molecular methods, and a detailed investigation of the causes of salpingitis/peritonitis by different detection methods, seems to be relevant spin-off investigations from the present work.

Regarding the pathogenic potential and the possibilities of separating strains of *Gallibacterium* according to virulence, this remains to be studied. However, more and more information emerges on other species of the family *Pasteurellaceae* Pohl 1981, like *Pasteurella multocida*, which is regarded as primary pathogen, from which it becomes increasingly apparent that the pathogenic potential of different strains can vary considerably (Heddleston, 1962; Wilkie *et al*., 2000; Petersen *et al*., 2001). Evidently, setting up limits for what can be considered as a pathogen versus a commensal is not straight forward and most often pathological lesions, morbidity and mortality are a result of an extremely complex interaction between several host and bacterial derived factors (Falkow, 1997).

We attempted to characterize the genetic element responsible for the haemolytic phenotype observed in certain *Gallibacterium* strains. Our focus on haemolysin as a putative virulence

factor was based on a qualified guess. Haemolysins/cytolysins, particularly of the RTX type, have been identified to represent virulence factors in several other members of *Pasteurellaceae* Pohl 1981 (Frey and Kuhnert, 2002). Unfortunately, our attempts did not succeed as we ended up identifying a gene possibly encoding FNR, which is able to activate the silent haemolysin SheA and confer a haemolytic phenotype of *E. coli* used as cloning host in our studies (Chapter 7). Future work will have to be done to elucidate which factors there may play a role in the pathogenesis of *Gallibacterium.*

Characterization and functional analysis of virulence factors is a complicated task, which may be complicated even further by the fact that some factors may only be expressed *in vivo* and during a short period of time (Moxon and Tang, 2000). However, whole-genome sequencing and expression profiling are powerful techniques that have permitted identification and analysis of factors putatively important for virulence (Moxon, 1997; Conway and Schoolnik, 2003). Several methods have recently been developed for these purposes and have contributed to the understanding of bacteria-host interaction of related species, including *A. pleuropneumoniae* and *P. multocida* (Fuller *et al*., 1999; Hunt *et al*., 2001). In spite of the great opportunities offered by these methods, there are still major issues to be addressed and here is evaluation of virulence factor expression *in vivo,* including where and when different factors are up or down regulated in different tissues and what impact host specificity has on the expression of virulence factors during infection of different hosts are some of the challenging issues that remains to be dealt with (Moxon and Tang, 2000; Boyce *et al*., 2002). The reasons for this are many, often there are not suitable infection models available, in other cases the technologies to assess large scale gene expression have not been adapted to the more complicated environment in the host.

In the case of *Gallibacterium*, a whole-genome approach has a long way to go. Besides the 16S rRNA sequence, only very little of the *Gallibacterium* genome has been sequenced and a whole-genome approach was evidently beyond the scope of this project. A whole-genome approach probably would contribute considerably to the understanding of processes of life of *Gallibacterium* in general and virulence in particular. However, in accordance with some of the points from the discussion of population genetics above, it also seem necessary to consider the actual outcome of a whole-genome approach, when applied to a single or a few strains of *Gallibacterium*. Obviously, the majority of genes and their regulation would be identical upon comparison of different strains, however, given the indicated heterogeneity with regards to preferred hosts and niches within these etc. it may well be in the minor differences that the really interesting features are hidden. In other words, the genome sequence of a single *Gallibacterium* isolate most probably will not be adequate for answering

a whole array of questions related to the specific interaction between representatives from vastly different environments and their host as this single isolate will not be able to account for that.

Consequently, it seems evident that there is a constant need for putting information gathered from detailed work based upon single isolates into a population genetic perspective.

The work in the present thesis aimed to account for this need and as may be deduced from the main conclusions outlined below, it has contributed with novel information regarding taxonomy, epidemiology and bacteria-host interaction of *Gallibacterium* infections in chickens.

From the work in the present thesis it can be concluded that:

- Bacteria previously classified as *Actinobacillus salpingitidis*, avian *Pasteurella haemolytica* and *P*. *anatis* belong to a new genus in the family of *Pasteurellaceae* Pohl 1981, named *Gallibacterium* and comprising one species, *G. anatis* and two additional genomospecies.
- The presence of haemolytic *Gallibacterium* spp. is widely distributed in most Danish chicken production systems and that infection of flocks on farm level is dependent on the biosecurity maintained in the individual flock.
- Vertical transmission is not a likely mode of dissemination of *Gallibacterium.*
- Natural populations of *Gallibacterium* primarily have a clonal population structure and that there may be clonal lineages adapted to different anatomical sites within the individual chicken.
- *Gallibacterium* can be detected and identified specifically by fluorescent 16S rRNA *in situ* hybridization, which enables evaluation of the spatial distribution of the bacteria within the infected host tissue and furthermore may be a more sensitive detection method than the current culture dependent techniques.
- *Gallibacterium* can be regarded as a secondary pathogen in chickens. At least certain strains seem capable of promoting severe morbidity and even mortality in the immunosuppressed host. Intraperitoneally inoculation furthermore evoked lesions similar to the findings from spontaneously diseased chickens.
- *Gallibacterium* possess a gene, named *fnrG*, a homologue to *fnr* in *Escherichia coli*, that encodes a transcriptional regulator capable of activating the silent haemolysin, SheA, which confers a haemolytic phenotype in *E. coli*.

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