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Genetic diversity of *Gallibacterium* isolates from California turkeys

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Genetic diversity of *Gallibacterium* isolates from California turkeysA. M. Bojesen^{1*} & H. L. Shivaprasad²**Abstract**

The aim of the present study was to investigate the genetic diversity of *Gallibacterium* isolates recovered from lesions in turkeys. *Gallibacterium* has been isolated from various bird species including turkeys, but no large investigations have yet been made to characterise isolates from turkeys genetically. Therefore we genotyped 53 *Gallibacterium* isolates obtained from turkeys between 1998 and 2004. Fifty isolates originated from 29 different flocks in California and the remaining three came from three German turkey flocks. All were recovered from birds with lesions, mainly in the upper respiratory tract. Five chicken isolates from California and five *Gallibacterium* reference strains were also included. Amplified fragment length polymorphism analysis demonstrated substantial genetic diversity among the *Gallibacterium* isolates. However, we also demonstrated that some *Gallibacterium* clones were present in consecutive rotations at the same farm during the entire six-year observation period and were present in different flocks from different farms. Similarly, the same clone was identified from two of the three German flocks. Further investigation of the spread of *Gallibacterium* between turkey flocks, including infections acquired from chickens or wild birds should be carried out.

Introduction

The taxonomy of organisms previously reported as [*Actinobacillus*] *salpingitidis*, avian [*Pasteurella*] *haemolytica* or [*P.*] *anatis* has recently been reinvestigated leading to formation of a new genus, *Gallibacterium* (Christensen *et al.*, 2003). The importance of *Gallibacterium* as a pathogen is not fully understood although a number of reports have indicated a pathogenic potential. Isolates have been recovered from mixed infections and in pure culture from a range of pathological lesions in poultry, including septicaemia, oophoritis, follicle degeneration, salpingitis, peritonitis, enteritis and respiratory tract lesions (Kjos-Hanssen, 1950; Kohlert, 1968; Mráz *et al.*, 1976; Gerlach, 1977; Matthes & Hanschke, 1977; Mushin *et al.*, 1980; Bisgaard & Dam, 1981; Shaw *et al.*, 1990; Mirle *et al.*, 1991; Bojesen *et al.*, 2003a; Jordan *et al.*, 2005).

Bacteria belonging to the 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 *et al.*, 1980; Bisgaard, 1993). Most studies of this organism have been in chickens and isolates obtained from other hosts have not been characterized to any great extent. A recent study reporting the occurrence of *Gallibacterium anatis* biovar *haemolytica* in California diagnostic avian specimens during 1994 to 2003 stated that *G. anatis* bv. *haemolytica* was isolated from a total of 152 out of 3609 meat turkeys subjected to *post mortem* analysis (Zellner *et al.*, 2004). However, little is known about the epidemiological aspects of *Gallibacterium* infections in turkeys at farm, flock or at host level. Genotypic methods involving characterization of the whole genome such as pulsed field gel electrophoresis (PFGE) and amplified fragment

length polymorphism (AFLP) have been used successfully to clarify epidemiological aspects of *Gallibacterium* infections in chicken flocks but isolates from turkeys have never been investigated on a large scale (Bojesen *et al.*, 2003; Christensen *et al.*, 2003). AFLP and PFGE represent highly reproducible and discriminatory genotyping methods allowing identification of clonal lineages in bacterial populations (Vos *et al.*, 1995; Spratt & Maiden, 1999).

Consequently, the aim of the present study was to characterize a collection of *G. anatis* *bv. haemolytica* isolates from turkeys by AFLP and evaluate population dynamics over time and within natural populations.

Materials and Methods

Bacterial strains. Fifty-three *Gallibacterium anatis* *bv. haemolytica* isolates from turkeys were characterized in this study. The California isolates were isolated between 1998 and 2004 from a total of 29 flocks owned by five different operations. An additional five isolates from chickens, also from California, were included for comparison. The chicken isolates originated from five epidemiologically unrelated flocks and were isolated in 2004. All isolates were retrieved from accessions in the California Animal Health and Food Safety Laboratory System. The remaining three isolates were obtained from three German turkey flocks. Five *Gallibacterium* reference strains (CCM5974, CCM5975, CCM5976, F149^T, 12656-12), including the type strain, were also included.

All the field strains were isolated from lesions, mainly in the upper respiratory tract. Other agents were also identified in most of the cases, the most prevalent being

Ornitobacterium rhinotracheale (six cases), *Bordetella avium* (six cases), *Escherichia coli* (five cases), *Pasteurella multocida* (one case), *Mycoplasma* spp. (one case) or a mixed bacterial population mainly composed of two or more of the above agents (24 cases) (Zellner *et al*, 2004). Swabs were plated on blood agar base supplemented with 5% bovine blood and the plates incubated overnight at 37°C in plastic bags. Circular smooth, shiny greyish raised colonies with a diameter of 1.0-2.0 mm after 24 h at 37°C and appearing strongly β -haemolytic, opaque, with a butyrous consistency were sub-cultured on blood agar plates to obtain pure cultures. Frozen stock cultures were subsequently made from cultures in Heart Infusion Broth (HIB; Difco, Detroit, MI.) incubated overnight at 37°C. Seven hundred microlitres of HIB culture were mixed with 300 μ l sterile 50% (v/v) glycerol and stored at -80°C.

Bacterial identification. All isolates were subjected to phenotypic characterization in accordance with Bisgaard (1982) and Christensen *et al.* (2003) and genotypic identification by the recently described *Gallibacterium*-specific PCR (Bojesen *et al.*, in press). This PCR is based on primers targeting the 16S and 23S rRNA genes generating two or three amplicons depending on the number of different ribosomal operons present. An internal amplification control based on the *rpoB* gene is also a part of the PCR protocol.

Genotyping by AFLP. All isolates were characterized as described by Bojesen *et al.* (2003b). Briefly, chromosomal DNA was extracted from cell pellets of overnight cultures in HIB by the use of Easy-DNA kit™ (Invitrogen, Carlsbad, CA, USA). The AFLP reactions were based on the non-selective *Bg*/II primer (FAM-5'GAGTACACTGTCGATCT 3') and the non-selective *Bsp*DI primer (5'

GTGTACTCTAGTCCGAT 3') and used to amplify the fragments subsequent to restriction digestion and ligation to their corresponding adaptors. Amplification products were detected on an ABI 377 automated sequencer (PE Biosystems). Each lane included internal-lane size standard labelled with ROX dye (Applied Biosystems) and GeneScan 3.1 fragment analysis software (Applied Biosystems) was used for fragment size determination and pattern analysis. AFLP profiles comprising fragments in the size range 50-500 bp were considered for numerical analysis with the program GelCompar II (Applied Maths, Kortrijk, Belgium). Normalised AFLP fingerprints were compared using the Dice similarity coefficient with a 0.5% optimization and 0.1% position tolerance, and clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA).

Results and Discussion

All included turkey isolates were β -haemolytic and classified with *Gallibacterium* based on the phenotypic characters tested. Similarly, *Gallibacterium*-specific amplicons at the expected sizes of 780 and 1060 bp were generated by PCR in all but one isolate (F0101711; data not shown). In addition, an intermediate size amplicon of ~900 bp was present in all isolates. Only the small and intermediate sized amplicons were present in F0101711. An amplicon of intermediate size indicating the existence of a third size ribosomal operon has been described previously by Christensen *et al.* (2003), however based on the previous and present results this operon does not seem to occur very frequently in *Gallibacterium* (Bojesen *et al.*, in press). The internal amplification control based on the *rpoB* gene was positive in all samples. The

phenotypic and genotypic results from the current investigation strongly indicate that all of the included isolates have been correctly identified as *Gallibacterium*.

The AFLP procedure was repeated only for those isolates (approximately 5%) that generated fingerprints that did not allow proper analysis in the first run. Repeat reactions were deemed unnecessary for others as our earlier study using the same AFLP protocol had a reproducibility of 97.5%, indicating that it was very robust (Bojesen *et al.*, 2003b).

A total of 41 individual *Gallibacterium* clones were identified among the 63 isolates using the 90% Dice similarity cut-off. This cut-off limit has been used in previous studies employing AFLP to assess the genetic diversity of *Gallibacterium* and other bacteria (Janssen *et al.*, 1997; Savelkoul *et al.*, 1999; Bojesen *et al.*, 2003b). Of the 41 clones, 33 appeared as single isolates whereas the remaining eight were part of clonal complexes as defined as groups of identical ($S_D \geq 90\%$) bacterial isolates (Figure 1). Three of the complexes contained from three to nine identical isolates. Interestingly, the same clone was isolated at different time points from operation A over the entire observation period from 1998 to 2004 (Figure 1). A similar picture was seen with the German turkey isolates where two flocks were infected with the same clone.

One of the Californian clones was isolated in one case from turkeys originating from six different flocks owned by two different operations (Figure 1). Strict biosecurity, including hygiene barriers and visitor clearing was practiced in all the operations included in our investigation although none of them operated on an 'all in all out' basis. For example, there were eight to 15 houses on each farm and the individual houses contained birds of different ages. Each farm was serviced by two or three workers, who did not visit flocks on any other farms. However, there was traffic

between farms by trucks carrying feed, live and dead birds. In addition, all farms were located in areas that were densely populated with commercial poultry including chickens and turkeys.

Gallibacterium organisms are not expected to survive well in poultry houses between rotations due to the cleaning and disinfection procedures and their poor viability outside the host (Bisgaard, 1993), but spread of these organisms and other pathogens between flocks and farms, for example by the wind, remains a possibility. There are other examples of bacterial clones, as in the case of *Pasteurella multocida*, that have been transmitted between poultry from different production systems and between farmed and wild birds (Christensen *et al.*, 1998; Pedersen *et al.*, 2003).

In addition to isolation from various types of poultry *Gallibacterium* organisms have been isolated from different bird species kept in captivity and from wild birds (Bisgaard, 1993). However, virtually nothing is known about possible interchange of organisms between these different bird populations. Although the current study was focussed on *Gallibacterium* isolated from turkeys, a small number of isolates from chickens, also from California, were characterized by AFLP. Interestingly, the California chicken isolates that were included appeared to be related to the turkey isolates and in one case even grouped together with a turkey clone, indicating that there could be a link between the different production systems (Fig. 1).

The five *Gallibacterium* reference strains were only distantly related to the California isolates, but this was expected due to the large temporal and geographical separation between them. A previous study assessing the genetic diversity among *Gallibacterium* isolates from chickens found that typically only one clone was present in each flock (Bojesen *et al.*, 2003b). However, isolates from only two flocks were

characterized and characterization of isolates from more flocks is probably necessary to substantiate this observation.

In conclusion, we have demonstrated a high genetic diversity among *Gallibacterium* isolates from Californian turkeys. In addition, we have shown that some *Gallibacterium* clones were present in consecutive flocks on the same farm over six years and in different flocks at different farms. Further studies are required to provide more insight into clonal spread and persistence of *Gallibacterium*.

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Figure Legends

Figure 1

Dendrogram showing AFLP similarities (Dice coefficient) between the Gallibacterium isolates obtained from turkeys. The vertical dotted line at 90% similarity denotes the cut-off value for a clone. The shaded boxes indicate groups of isolates belonging to the same clonal complex. The year of isolation is indicated in the first two digits of the strains number i.e. 98 is 1998.

