universität wien

DISSERTATION

Titel der Dissertation

Characterization of selected members of the genera *Campylobacter*, *Arcobacter*, *Helicobacter* and *Gallibacterium* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), with applications in clinical diagnostics

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

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To my loving family, Elma and Asmir

This work was carried out from September 2007 to November 2010 at the Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine in cooperation with University of Vienna.

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Summary

In almost every aspect of microbiology, e.g. clinical studies, epidemiological investigations and taxonomy, the identification of a strain to species, and sometimes subspecies, or even variant level is required. Without accurate identification methods, the estimation of the prevalence and significance of different species in a given environment is not possible. Clinical management is often facilitated if the identity of a strain is known. Within the scope of this PhD thesis members of the following four genera were analysed by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS): the closely related genera *Campylobacter*, *Arcobacter* and *Helicobacter* and furthermore the genus *Gallibacterium.* The main focus of the present study was the rapid and accurate identification and differentiation of species recognized as human and/or animal pathogens, with applications in clinical diagnostics.

In general, all species used in this study provided unique and reproducible whole-cell spectra (fingerprints), contributing to identification and differentiation of the strains. Well-characterized reference bacteria were used to generate reference databases in the MALDI/Biotyper software, to be used for correct identification of clinical strains. These results were compared to the results obtained by molecular methods. In addition, for reproducibility of results different growth as well as storage conditions were tested which are relevant in a diagnostic laboratory.

Besides the most important thermophilic species of *Campylobacter* genus, *Campylobacter jejuni* and *Campylobacter coli* which have important significance as food-borne pathogens, as well as *Arcobacter butzleri* and *Helicobacter pullorum*, several other members of these genera were investigated. In addition, 144 clinical isolates were identified correctly within a short period of time.

The recently established genus *Gallibacterium* represents a phenotypically heterogeneous group, where identification of species belonging to this genus is difficult. Therefore, 66 reference species of *Gallibacterium* were analysed by MALDI-TOF MS and by sequencing 16S rRNA, *rpoB*, *recN* and *infB* genes of some strains. Moreover, MALDI-TOF MS/Biotyper correctly identified 184 *Gallibacterium anatis* isolated from different organs from layers. Remarkably, MALDI-TOF MS revealed different clonal lineages of *G. anatis* between different flocks. Altogether, the ability of MALDI-TOF MS to be used in diagnostic laboratories is discussed.

Zusammenfassung

In nahezu allen Bereichen der Mikrobiologie, insbesondere im Rahmen von klinischen Studien, epidemiologischen Untersuchungen, und Taxonomie, ist die Identifizierung eines Bakterienstammes bis hin zu Spezies, Subspezies oder manchmal sogar Variante erforderlich. Ohne genaue Identifizierungsmethoden können in vielen Fällen Prävalenz und Bedeutung verschiedener Bakterienarten nicht bestimmt bzw. abgeschätzt werden. Im Rahmen der vorliegenden Dissertation wurden Isolate folgender vier Genera mittels Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Massenspektrometrie (MALDI-TOF-MS) analysiert: die eng miteinander verwandten Gattungen *Campylobacter*, *Arcobacter*, *Helicobacter* sowie die Gattung *Gallibacterium*. Der Schwerpunkt der Arbeit lag auf der schnellen und genauen Identifizierung und Differenzierung von Spezies dieser Gattungen, die als Krankheitserreger bei Mensch und/oder Tier von Bedeutung sind.

Alle in dieser Studie verwendeten Bakterienarten generierten jeweils einzigartige und reproduzierbare Spektren, die zu einer eindeutigen Identifizierung und Differenzierung der Bakterienstämme führten. Eine Referenzdatenbank in der MALDI/Biotyper Software wurde mit Hilfe von Referenzstämmen erstellt, welche dann für die Identifizierung von Feldisolaten verwendet wurde. Die so gewonnenen Ergebnisse wurden mit den Ergebnissen molekulargenetischer Methoden verglichen. Um die Reproduzierbarkeit der Ergebnisse für den Einsatz im diagnostischen Labor zu untersuchen, wurden darüber hinaus Bakterienstämme, die unter verschiedenen Wachstums- und Lagerbedingungen gehalten wurden, getestet.

Neben den beiden wichtigsten thermophilen *Campylobacter* Arten, *Campylobacter jejuni* und *Campylobacter coli*, welche, zusammen mit *Arcobacter butzleri* und *Helicobacter pullorum*, als weltweit führende Erreger humaner gastrointestinaler Erkrankungen angesehen werden, wurden auch andere Arten dieser Gattungen mittels MALDI-TOF MS untersucht. Darüber hinaus wurden 144 klinische Isolate schnell und korrekt identifiziert.

Die relativ junge Gattung *Gallibacterium* stellt eine phänotypisch sehr heterogene Gruppe der, wodurch sich die Identifizierung und Differenzierung einzelner Spezies oft als sehr problematisch erweist. Daher wurden 66 Referenzstämme mittels MALDI-TOF MS analysiert. Von einigen dieser stämme wurden 16S rRNA, *rpoB*, *recN* und *infB* sequenziert. Außerdem war mit der MALDI-TOF Methode die korrekte

Identifizierung und Zuordnung von klonalen Stämmen zu den jeweiligen Legehennenherden von 184 *G. anatis* möglich. Dabei konnten klonale Verbindungen von Isolaten innerhalb und zwischen Beständen identifiziert werden. Zusammenfassend wird der Einsatz von MALDI-TOF MS im diagnostischen Labor diskutiert.

Contents

1. Introduction

1.1. Introduction to the genera *Campylobacter, Arcobacter, Helicobacter* and *Gallibacterium*

1.1.1. Taxonomy and characteristics

Genus *Campylobacter*

The genus *Campylobacter* (meaning 'twisted bacteria') belongs to the epsilon class of *Proteobacteria,* in the order *Campylobacteriales*. This genus was first proposed in 1963 by Sebald and Veron, and contained at that time just two species: *Campylobacter fetus* and *Campylobacter bubulus,* which is now known as *C. sputorum* biovar *sputorum*. Originally, campylobacters were described as members of the genus *Vibrio* (Sebald & Veron, 1963). Currently, the genus *Campylobacter* comprises of 24 species and 11 subspecies (Euzeby, 1997).

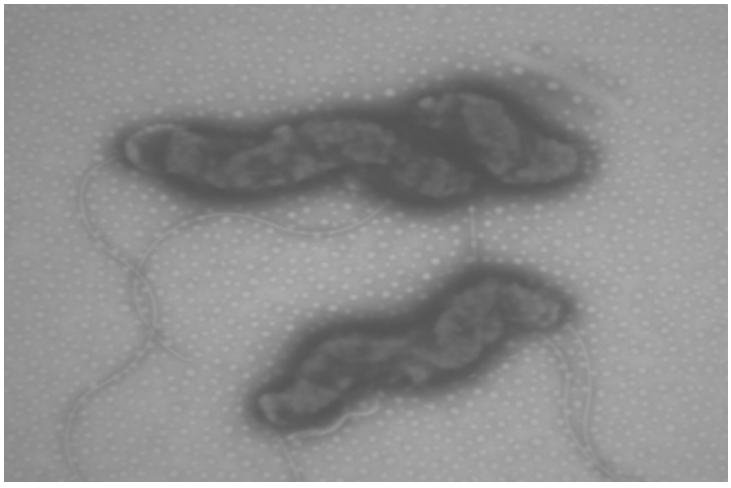

Figure 1. Scanning electron microscope image of *Campylobacter jejuni*, illustrating its corkscrew appearance and bipolar flagella. Source: Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine, Vienna, Austria.

All members of the genus *Campylobacter* are oxidase and catalase positive, curved, spiral or S-shaped Gram-negative bacteria, with tampered ends. The cells measure 0.2 to 0.5 μm wide and 0.5 to 8 μm long. The cells are actively motile by an unsheathed

polar flagellum located at one or both ends of the cell, which gives them a very characteristic “cork-screw” motility (Sebald & Veron, 1963) (Figure 1).

The bacteria are relatively fragile, and sensitive to environmental stress (e.g., 21% oxygen, drying, heating, disinfectants, and acidic conditions). The optimum growth temperature for thermophilic *Campylobacter* is 42°C, and for non-thermophilic *Campylobacter* 25°C-37°C in microaerophilic conditions. The organism does not grow at refrigeration conditions. This organism is comparatively slow growing even under optimum conditions. There is relatively little phenotypic information that can be used for classifying these bacteria. However, cell morphology and low guanine-plus-cytosine (G + C) content (28 to 38 mol %) of the deoxyribonucleic acid (DNA) are important criteria of this genus (Veron & Chatelai, 1973).

The type species of the *Campylobacter* genus is *C. fetus* subsp. *fetus*. However, the most important species of the genus is *C. jejuni* susp. *jejuni* often simply referred to as *C. jejuni* and represents the taxon first described by Jones *et al.* (1931) as ‘*Vibro jejuni*’ from bovine intestinal contents. *C. jejuni* susp. *jejuni* usually occurs as commensal in a wide range of animals hosts, including chickens, cattle, pigs, sheep, dogs and ostriches (Skirrow, 1994c). *Campylobacter lari* was first isolated from gulls and distinguished from the morphologically similar species *C. coli* and *C. jejuni* by virtue of its resistance to nalidixic acid (Skirrow & Benjamin, 1980). The species are ecologically, phenotypically and genetically diverse. Strains have been isolated from the intestinal tract of wild birds, poultry, cattle, shellfish and untreated water (Skirrow, 1994b; Aarestrup *et al.*, 1997).

Genus *Arcobacter*

In the late 1970s, Ellis and co-workers described spiral shaped bacteria which were isolated from aborted bovine and porcine foetuses (Ellis *et al.*, 1977; Ellis *et al.*, 1978). Their *Campylobacter*-like morphology, lack of fermentation activies, and rapid darting movement justified placement in the family *Campylobacteraceae* (Vandamme & Deley, 1991; Euzeby, 1992; Vandamme *et al.*, 1992a). In addition, the G + C content (29-34 mol%) initially calculated was within the range of the genus *Campylobacter* (Neill *et al.*, 1985). The genus name *Arcobacter* 'bow-shaped rod' was proposed, with *A.*

nitrofigilis as the type species and *A. cryaerophilus* and *A. butzleri* as additional species (Vandamme *et al.*, 1991c).

Members of the genus *Arcobacter* are described as aerotolerant Gram-negative curved rods, which are actively motile by means of a single unsheathed flagellum and grow under microaerophilic conditions (Neill *et al.*, 1985). *Arcobacter* are able to grow in atmospheric oxygen at 15°C, which clearly differentiates them from *Campylobacter* (Boudreau *et al.*, 1991). Similar to *Campylobacter*, *Arcobacter* species are metabolically inert, so few biochemical tests are available for differentiation. Phenotypic traits to differentiate species of *Arcobacter* (temperature dependent microaerophilic growth, catalase activity, nitrogen reduction, susceptibility to cadmium chloride, growth in the presence of 3.5% of NaCl and glycine, and growth on MacConkey agar) are limited (Neill *et al.*, 1978; Kiehlbauch *et al.*, 1991a; Vandamme *et al.*, 1992d). *A. cryaerophilus*, the most heterogeneous of the recognized species of *Arcobacter* is generally described as catalase positive, variable in nitrate reduction, and unable to grow in 1.5% NaCl (Vandamme *et al.*, 1992f). Currently, the genus *Arcobacter* consist of 9 species (Euzeby, 1997).

Genus *Helicobacter*

Marshall and Warren described spiral or curved bacilli isolated from human gastric mucosa (Marshall & Warren, 1984). The organism resembled *Campylobacter* in several respects, including curved morphology. Growth on rich media under micoaerophilic conditions, failure to ferment glucose, sensitivity to metronidazole and a G + C content of 34%. It was therefore first referred to as "pyloric *Campylobacter*" and later as *Campylobacter pylori*. After extensive analysis of the organism, major protein bands and fatty acids were different from those of *Campylobacter* species (Pearson *et al.*, 1984; Goodwin *et al.*, 1989), subsequent 16S rRNA sequence analysis confirmed that *C. pylori* should be excluded from the genus *Campylobacter* (Romaniuk *et al.*, 1987a). Thus, it was renamed *Helicobacter pylori*, the first member of the new genus *Helicobacter* (meaning 'a spiral rod') (Romaniuk *et al.*, 1987b).

Helicobacter are non-spore forming Gram-negative bacteria. The cellular morphology may be curved, spiral or fusiform, typically 0.2 to1.2 µm in diameter and 1.5 to 10.0 µm long. The spiral wavelength may vary with the age, the growth conditions, and the

species identity of the cells. In older cultures or those exposed to air, cells may become coccoid, the same is observed for *Campylobacter* and *Arcobacter* species. *Helicobacter* cells are motile, with a rapid cork screw like or slower wave like motion due to flagella activity. Strains of most species have bundles of multiple sheathed flagella with a polar or biopolar distribution. Other species have only a single polar or bipolar flagellum e.g. *H. pullorum* has nonsheathed flagella. The DNA G + C ratio of *Helicobacter* species generally ranges from 30 to 48 mol%.

Genus *Gallibacterium*

The family Pasteurellaceae Pohl 1981 was conceived to accommodate a large group of Gram-negative chemoorganotrophic, facultative anaerobic and fermentative bacteria including the genus *Gallibacterium* (meaning 'bacterium of chicken'). The genus *Gallibacterium* was established in 2003 (Christensen *et al.*, 2003e). Strains included in this genus were formerly classified as '*Actinobacillus salpingitidis*', avian [*Pasteurella*] *haemolytica*-like organisms and [*Pasteurella*] *anatis*. After extensive analysis of these strains by DNA-DNA hybridization (Piechulla *et al.*, 1985), rRNA-DNA hybridization (Deley *et al.*, 1990) and 16S rRNA sequencing comparison (Dewhirst *et al.*, 1993), a closer relationship was observed between '*A. salpingitidis*', [*Past.*] *haemolytica*-like and [*Past.*] *anatis*. To date genus *Gallibacterium* consist of four recognised species *Gallibacterium anatis*, *Gallibacterium melopsittaci*, *Gallibacterium salpingitidis*, and *Gallibacterium trehalosifermentans.*

All *Gallibacterium* species are Gram-negative, non-motile, rod-shaped or pleomorphic with cells occurring singly and in pairs. Colonies on bovine blood agar are mostly strong haemolytic, greyish, non-transparent, but eventually translucent at the periphery, with a butyrous consistency, smooth and shiny, circular, raised with an entire margin and 1-2 mm in diameter after 24-48 hrs. at 37 °C. Endospores are not formed. Growth is mesophilic and facultatively anaerobic or microaerophilic. Catalase, oxidase, and phosphatase positive, and nitrate is reduced (Christensen *et al.*, 2003d). The type strain F149^T (=ATCC 43329^T =NCTC 11413^T) of the type species is *G. anatis*, isolated from the intestinal tract of a duck. The G + C content range from 39.9 to 42.6 mol% and the genome size range from 1.6 to 2.1 GDa (Mutters *et al.*, 1985; Piechulla *et al.*, 1985). 16S rRNA gene sequence similarities of 93.2-94.8% between *G. anatis* type strain and the other species within *Gallibacterium* genus were reported (Bisgaard *et al.*, 2009)

However, 16S rRNA data indicated the existence of new *Gallibacterium* genomospecies 1 and 2 within the *Gallibacterium* genus (Christensen *et al.*, 2003f). In addition, Bisgaard *et al*. (2009) showed that 16S rRNA groups III (*Gallibacterium* genomospecies 3) and V (unnamed taxon group V) showed a maximum of 96.6% similarity with related taxa. Nonetheless, *G.* genomospecies 1 and 3 cannot be separated clearly from the other taxa by biochemical tests (Bisgaard *et al.*, 2009).

1.1.2. Human infections

The thermophilic *Campylobacter* species, particularly *C. jejuni*, *C. coli* and *C. lari*, cause campylobacteriosis, the most common human bacterial diarrhoea worldwide. In 2007, *Campylobacter* infections were the most frequently reported zoonotic disease in humans across the European Union with 200,507 cases (Figure 2). Epidemiological data suggest that contaminated products of animal origin, especially poultry, contribute significantly to campylobacteriosis (EFSA, 2009).

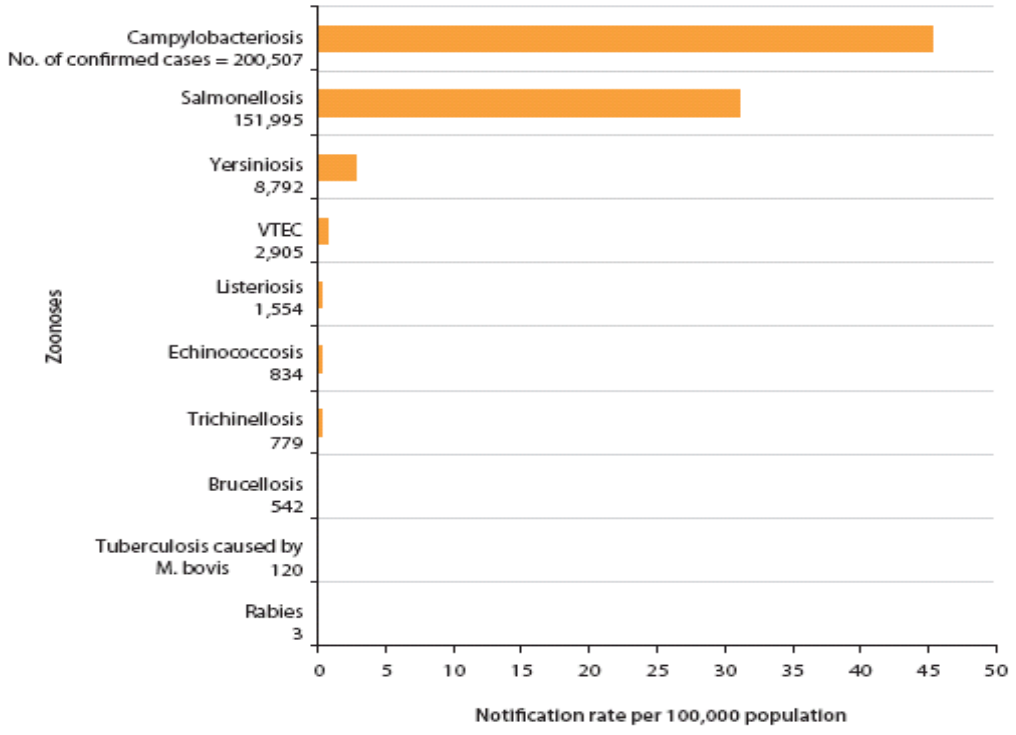

Figure 2. The reported notification zoonoses rates in confirmed human cases in the EU, 2007. Source: European Food Safety Authority (EFSA); Trends and sources of zoonoses and zoonotic agents in the European Union in 2007.

In industrialised countries, infection by enteric *Campylobacter* usually manifests itself as inflammatory diarrhoea with severe cramping. The highest rate of infection is in young adults, and asymptomatic carriage is infrequent.

In contrast, in less-well-developed countries there is a high rate of asymptomatic carriage, clinical symptoms are usually seen in children and non-inflammatory, watery diarrhoea is commonly observed (Wooldridge & Ketley, 1997).

Some people may develop autoimmune diseases such as, Reactive Arthritis (Pope *et al.*, 2007), or Miller Fisher syndrome (Salloway *et al.*, 1996) following campylobacteriosis. Whereas others may develop a rare disease called the Guillain-Barré syndrome (GBS), an acute inflammatory polyneuropathy, most frequently documented in association with *Campylobacter jejuni* (Nielsen *et al.*, 2000) seen as complication in 1 of 1000 cases of campylobacteriosis. GBS affects the nerves of the body, beginning several weeks after the diarrhoeal illness, and that can lead to paralysis lasting for up to several weeks. Approximately 0.1% of campylobacteriosis cases develop into GBS after two to three weeks of infection (Rhodes & Tattersfield, 1982; Yuki, 1997; Spiller *et al.*, 2000). *C. fetus* susp. *fetus* may cause diarrhoea, abortion, bacteraemia, endocarditis, and meningitis in humans (Farrugia *et al.*, 1994; On *et al.*, 1996). *C. hyointestinalis* subsp. *hyointestinalis* has been associated with sporadic (Edmonds *et al.*, 1987) and outbreak (Salama *et al.*, 1992) cases of diarrhoea in humans.

Two *Arcobacter* species are frequently isolated in human clinical samples: *A. butzleri* and *A. cryaerophilus,* and are regarded as emerging food-borne pathogens (Vandamme *et al.*, 1992b; On *et al.*, 1995; Vandenberg *et al.*, 2004; Prouzet-Mauleon *et al.*, 2006).

A zoonotic potential of *Helicobacter pullorum* is confirmed due to isolation of the organism from patients with enteritis and diarrhea (Stanley *et al.*, 1994; Steinbrueckner *et al.*, 1997; Casswall *et al.*, 2010). The frequent finding of this microorganism on raw poultry suggests that it may be food-borne. *H. pullorum* may also be involved in pathogenesis of chronic liver and gallbladder disease in humans (Ananieva *et al.*, 2002; Pellicano *et al.*, 2004; Laharie *et al.*, 2009; Casswall *et al.*, 2010).

The *Gallibacterium* species have no public health significance.

1.1.3. Animal infections

C. jejuni is mainly isolated from poultry and poultry products. The infection of poultry with *C. jejuni* is not associated with any obvious clinical signs (Zhang, 2008). However, *C. jejuni* has been occasionally associated with abortion in cattle and sheep (Anderson *et al.*, 1983; Welsh, 1984; Varga *et al.*, 1990; Campero *et al.*, 2005; Sahin *et al.*, 2007). *Campylobacter coli* were first isolated from pigs affected with infectious dysentery (Doyle & Hutchings, 1946; Doyle, 1948). It remains a frequently encountered species in pigs, although it is generally not thought to be a cause of disease in these animals. *C. coli* may also be found in cattle, poultry, ostriches and dogs. It is occasionally associated with hepatitis in birds (Stephens *et al.*, 1998). *C. fetus* subsp. *fetus* may be found in the intestine of cattle and sheep. It is a recognized cause of sporadic abortion in these animals (Skirrow, 1994a). *C. hyointestinalis* subsp. *hyointestinalis* are predominantly of enteric origin, typified by strains originally isolated from the pig intestine (Gebhart *et al.*, 1983). *C. hyointestinalis* subsp. *hyointestinalis* has since been found in healthy (Atabay & Corry, 1998) and diarrhoetic (Diker *et al.*, 1990) cattle.

Arcobacter spp. have been frequently isolated from foods of animal origin, in particular *A. butzleri,* are more frequently isolated from poultry (Rinsoz *et al.*, 2009; Collado & Figueras, 2011). *Arcobacter* are recovered from abortions and enteritis in livestock (Vandamme *et al.*, 1992c; On *et al.*, 2002). *A. cryaerophilus* are recovered from aborted litters, the genital tract of sows, the prepuce of boars, and more often from infertile sows with vaginal discharge than from normal animals (Wesley, 1997; deOliveira *et al.*, 1997).

Enterohepatic *H. pullorum* has been isolated from the ceca and feces of subclinically infected chickens, as well as from the livers and intestinal contents of laying hens with vibrionic hepatitis (Stanley *et al.*, 1994; Zanoni *et al.*, 2007). High prevalence of *H. pullorum* has been reported in conventional and organic broiler farms but significantly less in free-range broiler farms (Manfreda *et al.*, 2011). Recently, *H. pullorum* has been reported to colonize intestines in turkeys (Zanoni *et al.*, 2010). *H. pamatensis* has been isolated from bird feces, but it is of unknown pathogenicity (Dewhirst *et al.*, 1994).

Gallibacterium anatis is a common organism of the upper respiratory and lower genital tract of poultry (Bisgaard, 1977; Mushin *et al.*, 1980). The bacterium has been reported worldwide from a broad host range among farmed and wild birds. It is potentially pathogenic for poultry and is mainly associated with lesions in the reproductive tract, including the ovary (Gerlach, 1977; Bisgaard & Dam, 1981; Neubauer *et al.*, 2009). *Gallibacterium* spp. were reported to be prevalent in the Danish chicken production systems that were characterized as having low to moderate biosecurity levels (Bojesen *et al.*, 2003b). Disease associated with this microorganism is related to decreased egg production and occasionally an increase in mortality. The pathogenic *G. anatis* isolates have the ability to lyse red blood cells (Christensen *et al.*, 2003c) and produce haemolysin, which is a type of RTX-toxin called GtxA (Kristensen *et al.*, 2010).

1.1.4. Review of selected identification methods

Bacterial classifications prior to 1960's were predominantly based on cell morphology, growth requirements, biochemical and immunological tests. Phenotypic tests remain a commonly used approach to identify bacteria, however inconsistency of phenotypic profiles and similar phenotypic characters, prevent accurate identification. Accurate identification of a strain is essential to facilitate effective clinical management, estimate the prevalence of a given species, or as a precursor to the effective performance and evaluation of various analyses. Identification involves the comparison of data obtained for an unknown strain with those of known taxa. An isolate is identified when phenotypic (e.g. biochemical tests, fatty acid or protein profiles) or genotypic (e.g. DNA fingerprinting, sequencing, PCR primers) data matches that determined for a defined taxon to an acceptable level. The efficacy of a given method depends on fundamental knowledge of the level of diversity that may be encountered for a particular analyte in a given taxon. Inadequate consideration of species diversity can result in strain misidentification and, if such misidentified strains are subsequently presented as novel taxa, the potential for error in future studies increases.

Campylobacter, Arcobacter and *Helicobacter* species

The clinical and economic importance of *Campylobacter*, *Arcobacter*, and *Helicobacter* species coupled to their taxonomic complexity has led to a wide range of phenotypic and genetic methods being developed to identify them. Most routine laboratories use

biochemical tests for identifying and differentiating members of *Campylobacter*, *Arcobacter* and *Helicobacter* genera, based on various schema (On, 1996c; On, 2001). Identification of all members by traditional means is hampered by difficulties in isolation, culture and weak reactions toward some of the phenotypic tests used for identification.

Whereas *Arcobacter* strains can be differentiated from thermophilic *Campylobacter* and *Helicobacter* strains by their ability to grow in air and at low temperature, there are no clear biochemical characteristics to separate most members of the genus *Helicobacter* from the genus *Campylobacter*. *Helicobacter pullorum* bears a closer resemblance to certain *Campylobacter* species notably *Campylobacter lari* and *Campylobacter coli* (Steinbrueckner *et al.*, 1997). A commercial *Campylobacter* identification kit (API Campy; API Biomérieux Ltd., France), compromises 21 tests and 18 taxa, has been reported to misidentify *Arcobacter butzleri* as *Arcobacter cryaerophilus* or *Helicobacter cinaedi* (Jacob *et al.*, 1993), and problems in identifying certain *C. coli* and *C. lari* strains (Huysmans *et al.*, 1995; Reina *et al.*, 1995). In addition, not all species are included in this system e.g. *H. pullorum*.

Other phenotypically based methods, such as cellular fatty acid profiling and immunodiffusion, show similar problems in distinguishing certain groups (On, 1996b). Although DNA-DNA hybridization is generally considered the reference method, it is not practical to implement this technique in a routine laboratory or to examine large numbers of strains in a reference laboratory. The fastidious growth characteristics of many *Helicobacter* species hamper the isolation of sufficient quantities of highly purified high molecular weight DNA required for DNA-DNA hybridization. The present definition of a bacterial species is empirically based, 'The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m' (Wayne *et al.*, 1987), and genus 'those species which are linked by DNA homology above 55% represent a genus' (Mutters *et al.*, 1985).

The closest genetic relative to *C. jejuni* is *C. coli*, with which DNA-DNA hybridization values of 21-63% have been described (Harvey & Greenwood, 1983; Vandamme *et al.*, 1997b). These two species have a similar host range, are phenotypically homogeneous

and differentiation between the two is problematic. The most common test from this purpose is hippurate hydrolysis, in which *C. coli* gives a negative result. However, with atypical hippurate-negative strains of *C. jejuni* essentially indistinguishable from those of *C. coli* in routine laboratories (Morris *et al.*, 1985). Additional tests such as growth on a minimal medium and alpha-haemolytic activity may be of some use but require stringent standardization and, like hippurate hydrolysis, do not provide unequivocal discrimination (On, 1996a). The 16S rRNA gene sequences of these taxa are highly similar (Alderton *et al.*, 1995; Vandamme *et al.*, 1997a).

The biochemical and physiological properties of 90 aerotolerant *Campylobacter* strains were examined by Neill and co-workers (Neill *et al.*, 1978). They proposed a single name, *Campylobacter cryaerophila*, for the organisms which were isolated from reproductive tracts and aborted foetuses of several species of farm animals, from animal faeces, and from the milk of cows with mastitis (Neill *et al.*, 1978). After an extensive DNA-DNA hybridization study and phenotypic analysis of atypical *C. cryaerophila* strains, a separate species "*Campylobacter butzleri*" that exhibited a level of DNA homology with *C. cryaerophila* of approximately 40% was proposed (Vandamme *et al.*, 1991b; Kiehlbauch *et al.*, 1991b). Partial 16S rRNA sequence analysis showed that *C. cryaerophila* and *C. nitrofigilis* exhibited a 68% homology with other *Campylobacter* and 87% homology with each other (Thompson *et al.*, 1988). Therefore, *Arcobacter* as new genus was proposed. *Arcobacter nitrofigilis* the nitrogen-fixing commensal of plants is the type species and exhibits an 86.9% 16S rRNA sequence homology with other species of *Arcobacter* (Mcclung *et al.*, 1983; Vandamme *et al.*, 1991a; Vandamme *et al.*, 1992e).

PCR-based assays involve the amplification of specific DNA segments by annealing complementary primer sequences in either side of the target DNA region and subsequently synthesizing the DNA sequence in between by use of a DNA polymerase. In principle, the amplification process allows for a high level of sensitivity since very low levels of target DNA can be replicated at an exponential rate, and the PCR assay can be designed with almost any level of specificity in mind. Sequence specific to genera, species, subspecies or even strain level can be targeted and amplified by PCR. However, in order to design a PCR assay sequence data must be available and the most

frequently used target for the design of species-specific test is the 16S rRNA gene and also widely used 23S rRNA gene.

Variation in the rRNA gene can be unpredictable and may affect the security of any identification on which it is based. Intervening sequences (IVSs) have been detected in 16S and 23S rRNA of some *Campylobacter*, *Arcobacter* and *Helicobacter* species (Linton *et al.*, 1994b; Dewhirst *et al.*, 2005; Tazumi *et al.*, 2009; Douidah *et al.*, 2010). Intervening sequences may vary considerably in size (range presently 37-377 bp) and base composition, and are not always found in every copy of the rRNA operon (Linton *et al.*, 1994a; Harrington & On, 1999c).

Intervening sequences pose problems for conventional PCR and PCR-RFLP assays. Their insertion into a primer annealing site will result in a false-negative result; insertion between the primer binding sites may alter the size of the detected product. Where molecular size is a key or characteristic feature, this can prove confusing or misleading. Since existing PCR-RFLP identification assays are designed only from 16S or 23S rRNA sequence data, and require the amplification of a much larger part of these genes, they tend to be more susceptible to IVS phenomena. If the IVS itself contains a site recognized by one of the restriction enzymes in the scheme, then the problem becomes even more complex.

Intervening sequence elements do not represent the only means by which rRNA genes may exhibit diversity. In some species, the level of sequence variation between strains can be extensive and not restricted to a specific region of the gene. At present, *C. hyointestinalis* (Harrington & On, 1999d) are known to exhibit such extensive variation in their 16S rRNA genes that similarities as low as 95.7% have been described between strains of the same species (Harrington & On, 1999b). Divergence in a primer annealing site has the potential to cause a false-negative result. Harrington and On (1999a) noted that variance in the 16S rRNA gene of *C. hyointestinalis* had the potential to affect binding of the forward primer of a PCR identification assay for this species (Linton *et al.*, 1996). Ribosomal RNA gene sequence conservation between closely related species also presents a problem. It is well established that the taxonomic resolution of the 16S rRNA gene can be inadequate for delineating closely related species (Stackebrandt & Goebel, 1994).

Multilocus sequence typing (MLST), in principle, is a technique for typing of multiple loci, using the DNA sequences (450-500 bp) of usually seven conserved housekeeping genes, which encode essential proteins (Maiden *et al.*, 1998). MLST is used to investigate the intraspecies population structure, and such investigations have been performed with few *Campylobacter*, *Arcobacter* and *Helicobacter* species (Dingle *et al.*, 2001; Raymond *et al.*, 2004; Miller *et al.*, 2009). A major strength of MLST is that unambiguous, portable data are generated that can easily be compared among laboratories (Aanensen & Spratt, 2005). However, to choose the right primers for MLST analysis, some knowledge of the bacterial genetic background is required. MLST appears best in population genetic study but it is expensive. Due to the sequence conservation in housekeeping genes, MLST sometimes lacks the discriminatory power to differentiate bacterial strains, which limits its use in epidemiological investigation (Cai *et al.*, 2002).

Genus *Gallibacterium*

Identification of *Gallibacterium* is at present best performed through phenotypic characterisation outlined by Christensen *et al.* (2003b). However, identification of all members is hampered by difficulties in isolation, culture and weak reactions toward some of the phenotypic tests used for identification. In addition, it is difficult to separate the non-haemolytic isolates from *Avibacterium gallinarum*, whereas separation of haemolytic isolates from other taxa (e.g. haemolytic *Actinobacillus* sp.) is less problematic. The genus *Gallibacterium* can be separated from other genera of *Pasteurellaceae* by differences in 14 parameters, however, separation from avian members of *Pasteurella sensu stricto* is only possible at the species level by at least two characters (Christensen *et al.*, 2003a). *Gallibacterium anatis* have been misclassified as *Pasteurella multocida* since ornithine decarboxylase and indole negative isolates of *P. multocida* subsp. *septic* have the same phenotype (Bojesen *et al.*, 2007).

The fluorescent in situ hybridization technique (FISH) based on fluorescent material-labelled oligonucleotides complementary to bacteria 16S rRNA was shown to identify the genus *Gallibacterium* in both culture and tissue samples by the use of the *Gallibacterium* genus specific probe, GAN850 (Bojesen *et al.*, 2003a). This means that this method can only differentiate *Gallibacterium* from non-*Gallibacterium* species.

Gallibacterium has a relatively short internal transcribed 16S to 23S rRNA gene sequence compared to other members of *Pasteurellaceae*, this principle was used in the *Gallibacterium* PCR (Bojesen *et al.*, 2007). At the time of PCR development, only one recognised species (*G. anatis*) and one suggested species *G.* genomospecies 1 was included in the *Gallibacterium* genus. The PCR identifies *Gallibacterium* strains and differentiates them from other genera within *Pasteurellaceae*.

Amplified fragment length polymorphism (AFLP) was first described in 1995 as a powerful DNA fingerprinting technique that incorporates aspects of PCR and RFLP (Vos *et al.*, 1995). AFLP is advantageous because it can be used to type organisms for which very little or no sequence data are available. Bojesen *et al*. (Bojesen *et al.*, 2003c) showed that the AFLP typing method is useful for distinguishing individual but closely related *Gallibacterium anatis* clones, thus enabling recognition of specific pathogenic clonal lineages.

1.2. Microorganism identification by MALDI-TOF MS

The idea of rapid microorganism identification using matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) dates back to the mid 1990s. However, since the 1970s laser irradiation is used to generate ions for mass spectrometric analysis (Vastola *et al.*, 1970; Hillenkamp *et al.*, 1975; Posthumus *et al.*, 1978). Laser desorption ionisation (LDI) is based on the direct energy transfer to the sample by absorption of the analyte molecule. Due to the different spectral absorptions at the applied laser wavelength, only highly absorbing molecules could be detected, whereas ionization of none or less absorbing molecules was accompanied by extensive fragmentation. Moreover, the thermal degradation did not allow desorption and ionization of larger molecules as intact entities, therefore the accessible molecular mass range of LDI was below 2 kDa.

In the 1980s, Karas and Hillenkamp (1988) as well as Tanaka (1988) were able to overcome these limiting factors by embedding the analyte molecules into a highly UV absorbing matrix. Using the matrix as energy mediator controllable and efficient energy transfer was obtained and thermal degradation of the analyte molecules caused by

excessive energy could be avoided, mainly due the short time frame (low picoseconds range) of the laser pulse.

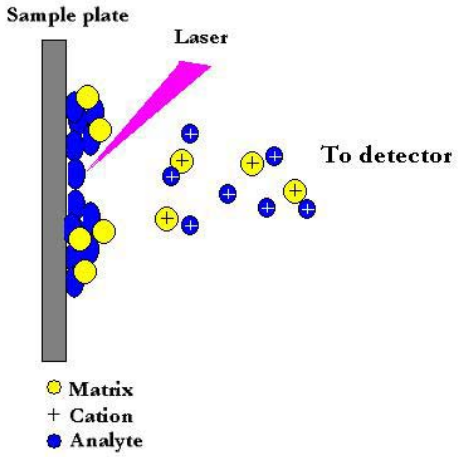

Figure 3. MALDI-TOF MS sample ionization

Since the introduction of MALDI-MS, numerous matrix compounds were investigated. Beside universal characteristics, low molecular weight, lack of chemical reactivity, solubility in analyte compatible solvents and vacuum stability, MALDI matrices provide several essential functions (Dreisewerd, 2003). The most important one is the high spectral absorption of the existing laser wavelength, allowing controllable and efficient energy transfer to the analyte and thus optimizing desorption/ionization process. Furthermore, the matrix separates the analyte molecules preventing analyte aggregation and clustering (Figure 3). The analyte as well as the matrix substance are dissolved in identical or at least compatible solvents and in proportion, so that, homogeneous distributed matrix/analyte crystal layers are formed, hence, reproducible mass spectra. After successful desorption and ionization of the analyte, the ions have to be accelerated and separated by a mass analyzer. Mass analysis by TOF analyzer is based on the fact that the time the ions need to pas the drift tube and reach the detector is directly correlated with their mass to charge (m/z) ratio.

A TOF spectrum is obtained by recording the signal intensity as a function of time. MALDI MS mostly provides singly charged ions facilitating data interpretation over wide mass range, thus m/z information can be obtained relative easily. Furthermore, it

offers a high tolerance against salts and detergents as well as speed, accuracy and the possibility of automation.

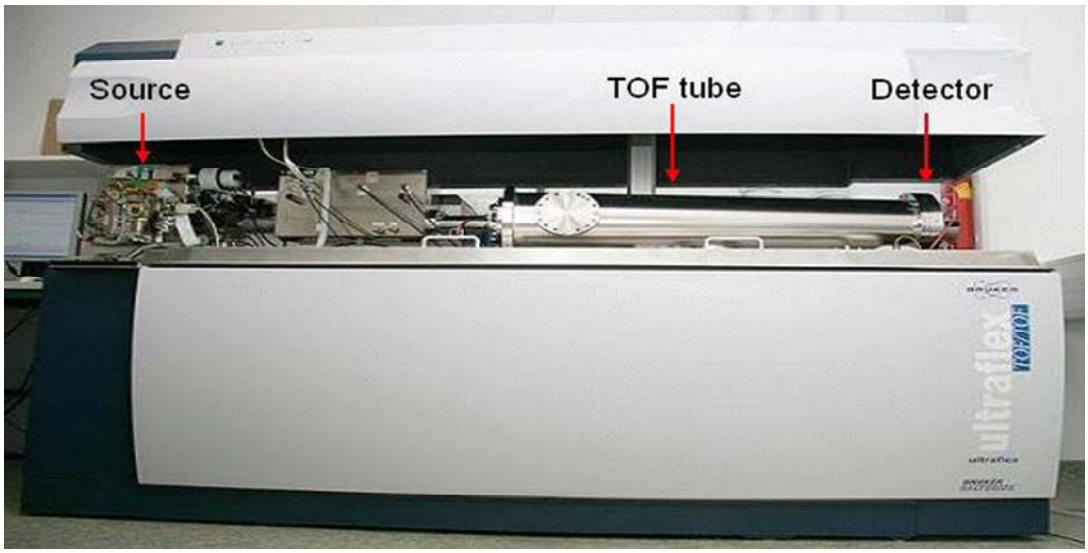

Figure 4. Bruker Ultraflex TOF/TOF II

Nowadays this technique, called vacuum matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), is widely used for the characterization of biopolymers, proteins and peptides (Hillenkamp *et al.*, 1991; Chaurand *et al.*, 1999). As well as oligosaccharides (Dell & Morris, 2001; Harvey, 2008), synthetic polymers (Bahr *et al.*, 1992; Nielen, 1999; Montaudo *et al.*, 2006), nucleic acids (Kirpekar *et al.*, 1995), and, profiling and imaging mass spectrometry of tissue sections (Chaurand *et al.*, 2006; Reyzer & Caprioli, 2007; Cornett *et al.*, 2007).

For fast microorganism identification using MALDI MS numerical methods are developed, these methods rely on some sort of database to which an unknown sample can be compared e.g. (1) a library of MALDI MS signatures constructed from spectra of known sample, (2) a library of proteins generated from one of the publicly available genomic and/or proteomic databases. An unknown sample spectrum is compared to signatures in the reference library, and a score for each comparison is generated. The sample contents are identified based on the score.

Recently, MALDI-TOF MS was reported as emerging technology for identification of bacteria (Claydon *et al.*, 1996; Grosse-Herrenthey *et al.*, 2008), fungi (Marklein *et al.*,

2009; De Respinis *et al.*, 2010), viruses (Luan *et al.*, 2009; La Scola *et al.*, 2010), insects (Perera *et al.*, 2005b), and nematodes (Perera *et al.*, 2005a).

The application of MALDI-TOF MS for bacteria identification has led to numerous encouraging results. Different species of bacteria yield mass spectra that display considerable variations (Holland *et al.*, 1996; Arnold & Reilly, 1998; van Baar, 2000). Bacteria of the same genus but different species exhibit mass spectra having both similarities and differences, just as one might anticipate in a fingerprinting method.

The drawback of MALDI-TOF MS is the requirement of pure bacterial cultures before any steps are taken for identification. Individual bacteria must generally be isolated from other cells and grown for one to five days to obtain pure cultures before identification, usually performed on selective media (e.g. modified charcoal cefoperazone deoxycholate agar (mCCDA) is used to isolate *Campylobacter* species). The traditional bacteriological techniques of streaking agar plates and serial dilution of liquids are still extremely useful for obtaining pure cultures. Because the time required for growth of pure cultures delays identification, serological and molecular techniques that bypass this step are often employed.

References

1. **Aanensen, D. M. & Spratt, B. G. (2005).** The multilocus sequence typing network: mlst.net. *Nucleic Acids Res* **33,** W728-W733.

2. **Aarestrup, F. M., Nielsen, E. M., Madsen, M. & Engberg, J. (1997).** Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from humans, pigs, cattle, and broilers in Denmark. *Antimicrob Agents Chemother* **41,** 2244-2250.

3. **Alderton, M. R., Korolik, V., Coloe, P. J., Dewhirst, F. E. & Paster, B. J. (1995).** *Campylobacter hyoilei* sp-nov, associated with porcine proliferative enteritis. *Int J Syst Bacteriol* **45,** 61-66.

4. **Ananieva, O., Nilsson, I., Vorobjova, T., Uibo, R. & Wadstrom, T. (2002).** Immune responses to bile-tolerant *Helicobacter* species in patients with chronic

liver diseases, a randomized population group, and healthy blood donors. *Clinical and Diagnostic Laboratory Immunology* **9,** 1160-1164.

5. **Anderson, K. L., Hamoud, M. M., Urbance, J. W., Rhoades, H. E. & Bryner, J. H. (1983).** Isolation of *Campylobacter-jejuni* from an aborted caprine fetus. *J Am Vet Med Assoc* **183,** 90-92.

6. **Arnold, R. J. & Reilly, J. P. (1998).** Fingerprint matching of *E. coli* strains with matrix-assisted laser desorption ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. *Rapid Communications in Mass Spectrometry* **12,** 630-636.

7. **Atabay, H. I. & Corry, J. E. L. (1998).** The isolation and prevalence of campylobacters from dairy cattle using a variety of methods. *J Appl Microbiol* **84,** 733-740.

8. **Bahr, U., Deppe, A., Karas, M., Hillenkamp, F. & Giessmann, U. (1992).** Mass spectrometry of synthetic-polymers by uv matrix-assisted laser desorption ionization. *Anal Chem* **64,** 2866-2869.

9. **Bisgaard, M. (1977).** Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. characterisation of 213 strains. *Avian Pathol* **6,** 285-292.

10. **Bisgaard, M. & Dam, A. (1981).** Salpingitis in poultry II. prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. *Nordisk Vet Med* **33,** 81-89.

11. **Bisgaard, M., Korczak, B. M., Busse, H. J., Kuhnert, P., Bojesen, A. M. & Christensen, H. (2009).** Classification of the taxon 2 and taxon 3 complex of Bisgaard within *Gallibacterium* and description of *Gallibacterium melopsittaci* sp nov., *Gallibacterium trehalosifermentans* sp nov and *Gallibacterium salpingitidis* sp nov. *Int J Syst Evol Microbiol* **59,** 735-744.

12. **Bojesen, A. M., Christensen, H., Nielsen, O. L., Olsen, J. E. & Bisgaard, M. (2003a).** Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA in situ hybridization. *J Clin Microbiol* **41,** 5167-5172.

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

14. **Bojesen, A. M., Torpdahl, M., Christensen, H., Olsen, J. E. & Bisgaard, M. (2003c).** Genetic diversity of *Gallibacterium anatis* isolates from different chicken flocks. *J Clin Microbiol* **41,** 2737-2740.

15. **Bojesen, A. M., Vazquez, M. E., Robles, F., Gonzalez, C., Soriano, E. V., Olsen, J. E. & Christensen, H. (2007).** Specific identification of *Gallibacterium* by a PCR using primers targeting the 16S rRNA and 23S rRNA genes. *Vet Microbiol* **123,** 262-268.

16. **Boudreau, M., Higgins, R. & Mittal, K. R. (1991).** Biochemical and serological characterization of *Campylobacter cryaerophila*. *J Clin Microbiol* **29,** 54-58.

17. **Cai, S., Kabuki, D. Y., Kuaye, A. Y., Cargioli, T. G., Chung, M. S., Nielsen, R. & Wiedmann, M. (2002).** Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. *J Clin Microbiol* **40,** 3319-3325.

18. **Campero, C. M., Anderson, M. L., Walker, R. L. & other authors (2005).** Immunohistochemical identification of *Campylobacter fetus* in natural cases of bovine and ovine abortions. *J Vet Med B Infect Dis Vet Public Health* **52,** 138-141.

19. **Casswall, T. H., Nemeth, A., Nilsson, I., Wadstrom, T. & Nilsson, H. O. (2010).** *Helicobacter* species DNA in liver and gastric tissues in children and adolescents with chronic liver disease. *Scandinavian Journal of Gastroenterology* **45,** 160-167.

20. **Chaurand, P., Luetzenkirchen, F. & Spengler, B. (1999).** Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry. *J Am Soc Mass Spectrom* **10,** 91-103.

21. **Chaurand, P., Norris, J. L., Cornett, D. S., Mobley, J. A. & Caprioli, R. M. (2006).** New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. *J Proteome Res* **5,** 2889-2900.

22. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003c).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

23. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003f).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

24. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003d).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

25. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003e).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

26. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003a).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

27. **Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003b).** 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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

28. **Claydon, M. A., Davey, S. N., EdwardsJones, V. & Gordon, D. B. (1996).** The rapid identification of intact microorganisms using mass spectrometry. *Nat Biothechnol* **14,** 1584-1586.

29. **Collado, L. & Figueras, M. J. (2011).** Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter*. *Clin Microbiol Rev* **24,** 174-192.

30. **Cornett, D. S., Reyzer, M. L., Chaurand, P. & Caprioli, R. M. (2007).** MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nature Methods* **4,** 828-833.

31. **De Respinis, S., Vogel, G., Benagli, C., Tonolla, M., Petrini, O. & Samuels, G. J. (2010).** MALDI-TOF MS of *Trichoderma*: a model system for the identification of microfungi. *Mycol Prog* **9,** 79-100.

32. **Deley, J., Mannheim, W., Mutters, R. & other authors (1990).** Interfamilial and intrafamilial similarities of ribosomal-RNA cistrons of the *Pasteurellaceae*. *Int J Syst Bacteriol* **40,** 126-137.

33. **Dell, A. & Morris, H. R. (2001).** Glycoprotein structure determination mass spectrometry. *Science* **291,** 2351-2356.

34. **deOliveira, S. J., Baetz, A. L., Wesley, I. V. & Harmon, K. M. (1997).** Classification of *Arcobacter* species isolated from aborted pig fetuses and sows with reproductive problems in Brazil. *Vet Microbiol* **57,** 347-354.

35. **Dewhirst, F. E., Paster, B. J., Olsen, I. & Fraser, G. J. (1993).** Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic-acid sequences. *Zbl Bakt-Int J Med M* **279,** 35-44.

36. **Dewhirst, F. E., Seymour, C., Fraser, G. J., Paster, B. J. & Fox, J. G. (1994).** Phylogeny of *Helicobacter* isolates from bird and swine feces and description of *Helicobacter pametensis* sp-nov. *Int J Syst Bacteriol* **44,** 553-560.

37. **Dewhirst, F. E., Shen, Z. L., Scimeca, M. S., Stokes, L. N., Boumenna, T., Chen, T. T., Paster, B. J. & Fox, J. G. (2005).** Discordant 16S and 23S rRNA gene phylogenies for the genus *Helicobacter*: Implications for phylogenetic inference and systematics. *Journal of Bacteriology* **187,** 6106-6118.

38. **Diker, K. S., Diker, S. & Ozlem, M. B. (1990).** Bovine diarrhea associated with *Campylobacter-hyointestinalis*. *Zbl Vet Med B* **37,** 158-160.

39. **Dingle, K. E., Colles, F. M., Wareing, D. R. A. & other authors (2001).** Multilocus sequence typing system for *Campylobacter jejuni*. *J Clin Microbiol* **39,** 14-23.

40. **Douidah, L., De Zutter, L., Vandamme, P. & Houf, K. (2010).** Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *Journal of Microbiological Methods* **80,** 281-286.

41. **Doyle, L. P. (1948).** The etiology of swine dysentery. *Am J Vet Res* **9,** 50-51.

42. **Doyle, L. P. & Hutchings, L. M. (1946).** A transmissible gastroenteritis in pigs. *J Am Vet Med Assoc* **108,** 257-259.

43. **Dreisewerd, K. (2003).** The desorption process in MALDI. *Chem Rev* **103,** 395-425.

44. **Edmonds, P., Patton, C. M., Griffin, P. M., Barrett, T. J., Schmid, G. P., Baker, C. N., Lambert, M. A. & Brenner, D. J. (1987).** *Campylobacter-hyointestinalis* associated with human gastrointestinal-disease in the United-States. *J Clin Microbiol* **25,** 685-691.

45. **EFSA (2009).** The community summary report on trends and sources of Zoonoses and Zoonotic agents in the European Union in 2007, *The EFSA Journal* (2009), 223.

46. **Ellis, W. A., Neill, S. D., Obrien, J. J., Ferguson, H. W. & Hanna, J. (1977).** Isolation of *Spirillum-Vibrio*-like organisms from bovine fetuses. *Vet Record* **100,** 451-452.

47. **Ellis, W. A., Neill, S. D., Obrien, J. J. & Hanna, J. (1978).** Isolation of *Spirillum*-like organisms from pig fetuses. *Vet Record* **102,** 106.

48. **Euzeby, J. P. (1992).** Bacterial systematics - changes of veterinary interest during 1991 - importance of veterinary-medicine. *Rev Med Vet-Toulouse* **143,** 13-28.

49. **Euzeby, J. P. (1997).** List of bacterial names with standing in nomenclature: A folder available on the Internet. *Int J Syst Bacteriol* **47,** 590-592.

50. **Farrugia, D. C., Eykyn, S. J. & Smyth, E. G. (1994).** *Campylobacter-fetus* endocarditis - 2 case-reports and review. *Clin Infect Dis* **18,** 443-446.

51. **Gebhart, C. J., Ward, G. E., Chang, K. & Kurtz, H. J. (1983).** *Campylobacter-hyointestinalis* (new species) isolated from swine with lesions of proliferative lleitis. *Am J Vet Res* **44,** 361-367.

52. **Gerlach, H. (1977).** Significance of *Pasteurella haemolytica* in poultry farms. *Prakt Tierarzt* **58,** 324-328.

53. **Goodwin, C. S., ARMSTRONG, J. A., CHILVERS, T., Peters, M., Collins, M. D., Sly, L., McConnell, W. & Harper, W. E. S. (1989).** Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen nov as *Helicobacter pylori* comb nov and *Helicobacter mustelae* comb nov, respectively. *Int J Syst Bacteriol* **39,** 397-405.

54. **Grosse-Herrenthey, A., Maier, T., Gessler, F., Schaumann, R., Bohnel, H., Kostrzewa, M. & Kruger, M. (2008).** Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). *Anaerobe* **14,** 242-249.

55. **Harrington, C. S. & On, S. L. W. (1999d).** Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *Int J Syst Bacteriol* **49,** 1171-1175.

56. **Harrington, C. S. & On, S. L. W. (1999c).** Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *Int J Syst Bacteriol* **49,** 1171-1175.

57. **Harrington, C. S. & On, S. L. W. (1999a).** Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *Int J Syst Bacteriol* **49,** 1171-1175.

58. **Harrington, C. S. & On, S. L. W. (1999b).** Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. *Int J Syst Bacteriol* **49,** 1171-1175.

59. **Harvey, D. J. (2008).** Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: An update covering the period 2001-2002. *Mass Spectrom Rev* **27,** 125-201.

60. **Harvey, S. M. & Greenwood, J. R. (1983).** Probable *Campylobacter-fetus* subsp fetus gastroenteritis. *J Clin Microbiol* **18,** 1278-1279.

61. **Hillenkamp, F., Karas, M., Beavis, R. C. & Chait, B. T. (1991).** Matrix-assisted laser desorption ionization mass-spectrometry of biopolymers. *Anal Chem* **63,** A1193-A1202.

62. **Hillenkamp, F., Unsold, E., Kaufmann, R. & Nitsche, R. (1975).** Laser microprobe mass analysis of organic materials. *Nature* **256,** 119-120.

63. **Holland, R. D., Wilkes, J. G., Rafii, F., Sutherland, J. B., Persons, C. C., Voorhees, K. J. & Lay, J. O. (1996).** Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **10,** 1227-1232.

64. **Huysmans, M. B., Turnidge, J. D. & Williams, J. H. (1995).** Evaluation of API Campy in comparison with conventional methods for identification of thermophilic campylobacters. *J Clin Microbiol* **33,** 3345-3346.

65. **Jacob, J., Lior, H. & Feuerpfeil, I. (1993).** Isolation of *Arcobacter butzleri* from a drinking water reservoir in Eastern Germany. *Zentralblatt fur Hygiene und Umweltmedizin* **193,** 557-562.

66. **Jones, F. S., Orcutt, M. & Little, R. B. (1931).** Vibrios (*Vibrio jejuni*, N.sp.) associated with intestinal disorders of cows and calves. *J Exp Med* **53,** 853-863.

67. **Karas, M. & Hillenkamp, F. (1988).** Laser desorption ionization of proteins with molecular masses exceeding 10000 daltons. *Anal Chem* **60,** 2299-2301.

68. **Kiehlbauch, J. A., Brenner, D. J., Nicholson, M. A., Baker, C. N., Patton, C. M., Steigerwalt, A. G. & Wachsmuth, I. K. (1991a).** *Campylobacter-butzleri* sp-nov isolated from humans and animals with diarrheal illness. *J Clin Microbiol* **29,** 376-385.

69. **Kiehlbauch, J. A., Plikaytis, B. D., Swaminathan, B., Cameron, D. N. & Wachsmuth, I. K. (1991b).** Restriction-fragment-length-polymorphisms in the ribosomal genes for species identification and subtyping of aerotolerant *Campylobacter* species. *J Clin Microbiol* **29,** 1670-1676.

70. **Kirpekar, F., Nordhoff, E., Kristiansen, K., Roepstorff, P., Hahner, S. & Hillenkamp, F. (1995).** 7-Deaza purine bases offer a higher ion stability in the analysis of DNA by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Communications in Mass Spectrometry* **9,** 525-531.

71. **Kristensen, B. M., Frees, D. & Bojesen, A. M. (2010).** GtxA from *Gallibacterium anatis*, a cytolytic RTX-toxin with a novel domain organisation. *Vet Res* **41,** 25.

72. **La Scola, B., Campocasso, A., N'Dong, R., Fournous, G., Barrassi, L., Flaudrops, C. & Raoult, D. (2010).** Tentative characterization of new environmental giant viruses by MALDI-TOF mass spectrometry. *Intervirology* **53,** 344-353.

73. **Laharie, D., Asencio, C., Asselineau, J. & other authors (2009).** Association between entero-hepatic *Helicobacter* species and Crohn's disease: a prospective cross-sectional study. *Alimentary Pharmacology & Therapeutics* **30,** 283-293.

74. **Linton, D., Dewhirst, F. E., Clewley, J. P., Owen, R. J., Burnens, A. P. & Stanley, J. (1994b).** Two types of 16S RNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains. *Microbiology-Uk* **140,** 847-855.

75. **Linton, D., Dewhirst, F. E., Clewley, J. P., Owen, R. J., Burnens, A. P. & Stanley, J. (1994a).** Two types of 16S RNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains. *Microbiology-Uk* **140,** 847-855.

76. **Linton, D., Owen, R. J. & Stanley, J. (1996).** Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Research in Microbiology* **147,** 707-718.

77. **Luan, J., Yuan, J., Li, X. H. & other authors (2009).** Multiplex detection of 60 hepatitis B virus variants by MALDI-TOF mass spectrometry. *Clin Chem* **55,** 1503-1509.

78. **Maiden, M. C. J., Bygraves, J. A., Feil, E. & other authors (1998).** Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 3140-3145.

79. **Manfreda, G., Parisi, A., Lucchi, A., Zanoni, R. G. & De, C. A. (2011).** Prevalence of *Helicobacter pullorum* in conventional, organic, and free-range broilers and typing of isolates. *Appl Environ Microbiol* **77,** 479-484.

80. **Marklein, G., Josten, M., Klanke, U. & other authors (2009).** Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. *J Clin Microbiol* **47,** 2912-2917.

81. **Marshall, B. J. & Warren, J. R. (1984).** Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1,** 1311-1315.

82. **Mcclung, C. R., Patriquin, D. G. & Davis, R. E. (1983).** *Campylobacter-nitrofigilis* sp-nov, a nitrogen-fixing bacterium associated with roots of *Spartina-alterniflora* Loisel. *Int J Syst Bacteriol* **33,** 605-612.

83. **Miller, W. G., Wesley, I. V., On, S. L. W., Houf, K., Megraud, F., Wang, G. L., Yee, E., Srijan, A. & Mason, C. J. (2009).** First multi-locus sequence typing scheme for *Arcobacter* spp. *Bmc Microbiology* **9,** doi:10.1186/1471-2180-9-196.

84. **Montaudo, G., Samperi, F. & Montaudo, M. S. (2006).** Characterization of synthetic polymers by MALDI-MS. *Prog Polym Sci* **31,** 277-357.

85. **Morris, G. K., Elsherbeeny, M. R., Patton, C. M., Kodaka, H., Lombard, G. L., Edmonds, P., Hollis, D. G. & Brenner, D. J. (1985).** Comparison of 4 hippurate hydrolysis methods for identification of thermophilic *Campylobacter* Spp. *J Clin Microbiol* **22,** 714-718.

86. **Mushin, R., Weisman, Y. & Singer, N. (1980).** *Pasteurella haemolytica* found in the respiratory-tract of fowl. *Avian Dis* **24,** 162-168.

87. **Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. (1985).** Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic-acid homology, with proposals for the new species *Pasteurella-dagmatis*, *Pasteurella-canis*, *Pasteurella-stomatis*, *Pasteurella-anatis*, and *Pasteurella-langaa*. *Int J Syst Bacteriol* **35,** 309-322.

88. **Neill, S. D., Campbell, J. N., Obrien, J. J., Weatherup, S. T. C. & Ellis, W. A. (1985).** Taxonomic position of *Campylobacter-cryaerophila* sp-nov. *Int J Syst Bacteriol* **35,** 342-356.

89. **Neill, S. D., Ellis, W. A. & Obrien, J. J. (1978).** Biochemical characteristics of *Campylobacter*-like organisms from cattle and pigs. *Res Vet Sci* **25,** 368-372.

90. **Neubauer, C., De Souza-Pilz, M., Bojesen, A. M., Bisgaard, M. & Hess, M. (2009).** Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders. *Avian Pathol* **38,** 1-7.

91. **Nielen, M. W. F. (1999).** Maldi time-of-flight mass spectrometry of synthetic polymers. *Mass Spectrom Rev* **18,** 309-344.

92. **Nielsen, E. M., Engberg, J., Fussing, V., Petersen, L., Brogren, C. H. & On, S. L. W. (2000).** Evaluation of phenotypic and genotypic methods for subtyping *Campylobacter jejuni* isolates from humans, poultry, and cattle. *J Clin Microbiol* **38,** 3800-3810.

93. **On, S. L. (1996c).** Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. *Clin Microbiol Rev* **9,** 405-422.

94. **On, S. L. (1996b).** Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. *Clin Microbiol Rev* **9,** 405-422.

95. **On, S. L. (1996a).** Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. *Clin Microbiol Rev* **9,** 405-422.

96. **On, S. L. W. (2001).** Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. *J Appl Microbiol* **90,** 1S-15S.

97. **On, S. L. W., Holmes, B. & Sackin, M. J. (1996).** A probability matrix for the identification of Campylobacters, Helicobacters and allied taxa. *J Appl Bacteriol* **81,** 425-432.

98. **On, S. L. W., Jensen, T. K., Bille-Hansen, V., Jorsal, S. E. & Vandamme, P. (2002).** Prevalence and diversity of *Arcobacter* spp. isolated from the internal organs of spontaneous porcine abortions in Denmark. *Vet Microbiol* **85,** 159-167.

99. **On, S. L. W., Stacey, A. & Smyth, J. (1995).** Isolation of *Arcobacter-butzler*i from a neonate with Bacteremia. *Journal of Infection* **31,** 225-227.

100. **Pearson, A. D., Ireland, A., Bamforth, J., Walker, C., Booth, L., Hawtin, P., Holdstock, G. & Millwardsadler, H. (1984).** Polyacrylamide-gel electrophoresis of spiral bacteria from the gastric antrum. *Lancet* **1,** 1349-1350.

101. **Pellicano, R., Touscoz, G. A., Smedile, A., Berrutti, M., Saracco, G., Repici, A., Ponzetto, A. & Rizzetto, M. (2004).** Prevalence of non-organ-specific autoantibodies in patients suffering from duodenal ulcer with and without *Helicobacter pylori* infection. *Digestive Diseases and Sciences* **49,** 395-398.

102. **Perera, M. R., Vanstone, V. A. & Jones, M. G. K. (2005a).** A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **19,** 1454-1460.

103. **Perera, M. R., Vargas, R. D. F. & Jones, M. G. K. (2005b).** Identification of aphid species using protein profiling and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Entomol Exp Appl* **117,** 243-247.

104. **Piechulla, K., Bisgaard, M., Gerlach, H. & Mannheim, W. (1985).** Taxonomy of some recently described avian *Pasteurella actinobacillus*-like organisms as indicated by deoxyribonucleic-acid relatedness. *Avian Pathol* **14,** 281-311.

105. **Pope, J. E., Krizova, A., Garg, A. X., Thiessen-Philbrook, H. & Ouimet, J. A. (2007).** *Campylobacter* reactive arthritis: A systematic review. *Seminars in Arthritis and Rheumatism* **37,** 48-55.

106. **Posthumus, M. A., Kistemaker, P. G., Meuzelaar, H. L. C. & Tennoeverdebrauw, M. C. (1978).** Laser desorption-mass spectrometry of polar non-volatile bio-organic molecules. *Anal Chem* **50,** 985-991.

107. **Prouzet-Mauleon, V., Labadi, L., Bouges, N., Menard, A. & Megraud, F. (2006).** *Arcobacter butzleri*: underestimated enteropathogen. *Emerg Infect Dis* **12,** 307-309.

108. **Raymond, J., Thiberge, J. M., Chevalier, C., Kalach, N., Bergeret, M., Labigne, A. & Dauga, C. (2004).** Genetic and transmission analysis of *Helicobacter pylori* strains within a family. *Emerg Infect Dis* **10,** 1816-1821.

109. **Reina, J., Ros, M. J. & Serra, A. (1995).** Evaluation of the API-Campy system in the biochemical identification of hippurate negative *Campylobacter* strains isolated from feces. *J Clin Pathol* **48,** 683-685.

110. **Reyzer, M. L. & Caprioli, R. M. (2007).** MALDI-MS-based imaging of small molecules and proteins in tissues. *Curr Opin Chem Biol* **11,** 29-35.

111. **Rhodes, K. M. & Tattersfield, A. E. (1982).** Guillain-Barre syndrome associated with *Campylobacter* infection. *Br Med J (Clin Res Ed)* **285,** 173-174.

112. **Rinsoz, T., Hilfiker, S. & Oppliger, A. (2009).** Quantification of thermotolerant *Campylobacter* in Swiss water treatment plants, by real-time quantitative polymerase chain reaction. *Water Environ Res* **81,** 929-933.

113. **Romaniuk, P. J., Zoltowska, B., Trust, T. J., Lane, D. J., Olsen, G. J., Pace, N. R. & Stahl, D. A. (1987b).** Campylobacter pylori, the spiral bacterium associated with human gastritis, is not a true Campylobacter sp. *J Bacteriol* **169,** 2137-2141.

114. **Romaniuk, P. J., Zoltowska, B., Trust, T. J., Lane, D. J., Olsen, G. J., Pace, N. R. & Stahl, D. A. (1987a).** Campylobacter pylori, the spiral bacterium

associated with human gastritis, is not a true Campylobacter sp. *J Bacteriol* **169,** 2137-2141.

115. **Sahin, O., Plummer, P., Sulaj, K. & other authors (2007).** An emerging strain of *Campylobacter jejuni* responsible for ovine abortion in the United States. *Zoonoses Public Health* **54,** 80.

116. **Salama, S. M., Tabor, H., Richter, M. & Taylor, D. E. (1992).** Pulsed-field gel-electrophoresis for epidemiologic studies of *Campylobacter-hyointestinalis* isolates. *J Clin Microbiol* **30,** 1982-1984.

117. **Salloway, S., Mermel, L. A., Seamans, M., Aspinall, G. O., Shin, J. E. N., Kurjanczyk, L. A. & Penner, J. L. (1996).** Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD(3). *Infect Immun* **64,** 2945-2949.

118. **Sebald, M. & Veron, M. (1963).** Teneur en bases de ladn et classification des *Vibrions*. *Ann Inst Pasteur* **105,** 897-910.

119. **Skirrow, M. B. (1994a).** Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *J Comp Pathol* **111,** 113-149.

120. **Skirrow, M. B. (1994b).** Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *J Comp Pathol* **111,** 113-149.

121. **Skirrow, M. B. (1994c).** Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *J Comp Pathol* **111,** 113-149.

122. **Skirrow, M. B. & Benjamin, J. (1980).** Differentiation of enteropathogenic *Campylobacter*. *J Clin Pathol* **33,** 1122.

123. **Spiller, R. C., Jenkins, D., Thornley, J. P., Hebden, J. M., Wright, T., Skinner, M. & Neal, K. R. (2000).** Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute *Campylobacter* enteritis and in post-dysenteric irritable bowel syndrome. *Gut* **47,** 804-811.

124. **Stackebrandt, E. & Goebel, B. M. (1994).** A place for DNA-DNA reassociation and 16S ribosomal-RNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44,** 846-849.

125. **Stanley, J., Linton, D., Burnens, A. P., Dewhirst, F. E., On, S. L. W., Porter, A., Owen, R. J. & Costas, M. (1994).** *Helicobacter pullorum* sp-nov - genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiol* **140,** 3441-3449.

126. **Steinbrueckner, B., Haerter, G., Pelz, K., Weiner, S., Rump, J. A., Deissler, W., Bereswill, S. & Kist, M. (1997).** Isolation of *Helicobacter pullorum* from patients with enteritis. *Scandinavian Journal of Infectious Diseases* **29,** 315-318.

127. **Stephens, C. P., On, S. L. W. & Gibson, J. A. (1998).** An outbreak of infectious hepatitis in commercially reared ostriches associated with *Campylobacter coli* and *Campylobacter jejuni*. *Vet Microbiol* **61,** 183-190.

128. **Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. & Yoshida, T. (1988).** Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **2,** 151-153.

129. **Tazumi, A., Kakinuma, Y., Misawa, N., Moore, J. E., Millar, B. C. & Matsuda, M. (2009).** Identification and characterization of intervening sequences within 23S rRNA genes from more than 200 *Campylobacter* isolates from seven species including atypical campylobacters. *Bmc Microbiology* **9,** doi:10.1186/1471-2180-9-256.

130. **Thompson, L. M., Smibert, R. M., Johnson, J. L. & Krieg, N. R. (1988).** Phylogenetic study of the genus *Campylobacter*. *Int J Syst Bacteriol* **38,** 190-200.

131. **van Baar, B. L. M. (2000).** Characterisation of bacteria by matrix-assisted laser desorption/ionisation and electrospray mass spectrometry. *Fems Microbiol Rev* **24,** 193-219.

132. **Vandamme, P. & Deley, J. (1991).** Proposal for a new family, *Campylobacteraceae*. *Int J Syst Bacteriol* **41,** 451-455.

133. **Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De, L. J. (1991a).** Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41,** 88-103.

134. **Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De, L. J. (1991b).** Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41,** 88-103.

135. **Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De, L. J. (1991c).** Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41,** 88-103.

136. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992e).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri*

comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

137. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992c).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri* comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

138. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992b).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri* comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

139. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992f).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri* comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

140. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992d).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri* comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

141. **Vandamme, P., Vancanneyt, M., Pot, B. & other authors (1992a).** Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter-butzleri* comb-nov and *Arcobacter-skirrowii* sp-nov, an aerotolerant bacterium isolated from veterinary specimens. *Int J Syst Bacteriol* **42,** 344-356.

142. **Vandamme, P., VanDoorn, L. J., AlRashid, S. T., Quint, W. G. V., VanderPlas, J., Chan, V. L. & On, S. L. W. (1997a).** *Campylobacter hyoilei* Alderton et al. 1995 and *Campylobacter coli* Veron and Chatelain 1973 are subjective synonyms. *Int J Syst Bacteriol* **47,** 1055-1060.

143. **Vandamme, P., VanDoorn, L. J., AlRashid, S. T., Quint, W. G. V., VanderPlas, J., Chan, V. L. & On, S. L. W. (1997b).** *Campylobacter hyoilei* Alderton et al. 1995 and *Campylobacter coli* Veron and Chatelain 1973 are subjective synonyms. *Int J Syst Bacteriol* **47,** 1055-1060.

144. **Vandenberg, O., Dediste, A., Houf, K. & other authors (2004).** *Arcobacter* species in humans. *Emerg Infect Dis* **10,** 1863-1867.

145. **Varga, J., Mezes, B., Fodor, L. & Hajtos, I. (1990).** Serogroups of *Campylobacter fetus* and *Campylobacter jejuni* isolated in cases of ovine abortion. *Zbl Vet Med B* **37,** 148-152.

146. **Vastola, F. J., Mumma, R. O. & Pirone, A. J. (1970).** Analysis of organic salts by laser ionization. *Org Mass Spectrom* **3,** 101-104.

147. **Veron, M. & Chatelai, R. (1973).** Taxonomic study of genus *Campylobacter* Sebald and Veron and designation of neotype strain for type species, *Campylobacter-fetus* (Smith and Taylor) Sebald and Veron. *Int J Syst Bacteriol* **23,** 122-134.

148. **Vos, P., Hogers, R., Bleeker, M. & other authors (1995).** Aflp - A new technique for DNA-fingerprinting. *Nucleic Acids Res* **23,** 4407-4414.

149. **Wayne, L. G., Brenner, D. J., Colwell, R. R. & other authors (1987).** Report of the Ad-Hoc-Committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37,** 463-464.

150. **Welsh, R. D. (1984).** *Campylobacter jejuni* abortion in a heifer. *J Am Vet Med Assoc* **185,** 549-551.

151. **Wesley, I. V. (1997).** *Helicobacter* and *Arcobacter*: Potential human foodborne pathogens? *Trends in Food Science & Technology* **8,** 293-299.

152. **Wooldridge, K. G. & Ketley, J. M. (1997).** *Campylobacter* host cell interactions. *Trends Microbiol* **5,** 96-102.

153. **Yuki, N. (1997).** Molecular mimicry between gangliosides and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Guillain-Barre syndrome and Miller Fisher syndrome. *J Infect Dis* **176,** S150-S153.

154. **Zanoni, R. G., Piva, S., Rossi, M., Pasquali, F., Lucchi, A., De, C. A. & Manfreda, G. (2010).** Occurrence of *Helicobacter pullorum* in turkeys. *Vet Microbiol*.

155. **Zanoni, R. G., Rossi, M., Giacomucci, D., Sanguinetti, V. & Manfreda, G. (2007).** Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. *Int J Food Microbiol* **116,** 168-173.

156. **Zhang, Q. (2008).** Diseases of Poultry 12th Edition., pp. 675-689. Edited by Y. M. Saif Blackwell, Iowa.

2. Aim of this thesis

The aim of this PhD thesis was to apply matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to certain bacteria recognized as human and/or animal pathogens. That is to establish a reference database enabling the correct identification and characterization of clinical samples.

Members of the following four genera: the closely related genera *Campylobacter*, *Arcobacter* and *Helicobacter* and furthermore the recently established genus *Gallibacterium* were characterized by MALDI-TOF MS. The main focus of the present study was the rapid and accurate identification and differentiation of species belonging to the four genera mentioned above, with applications in clinical diagnostics. Moreover, the ability of MALDI-TOF MS to be used in typing of defined bacterial population in comparison to genotypic methods, for enabling strain-specific identification. The overall efficacy of whole-cell MALDI-TOF MS methods for taxonomic classification can be judged based on comparison with accepted methods, thus, in each case the results of MALDI-TOF MS were compared to results obtained by additional molecular methods.

Bacteria respond rapidly to environmental changes. Changes in cellular processes occur very soon after changes when cells are stored, handled or cultured over different time periods prior to analysis. These changes result in rapid change in the protein profile. Thus, reproducibility of MALDI-TOF MS results were evaluated in response to different growth as well as storage conditions of bacteria, which are relevant in a diagnostic laboratory. Furthermore, because much smaller differences are anticipated at the strain level, strain level differences and spectral reproducibility have received much attention in order to generate reliable results.

3. Publications

The results and discussion of this thesis consists of three manuscripts focusing on the application of MALDI-TOF MS to certain bacteria recognized as human and/or animal pathogens. A number of important issues relevant in clinical diagnostics were addressed.

3.1. Species-specific identification and differentiation of *Arcobacter, Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis

Manuscript published in *Journal of Medical Microbiology*, 2010, (IF 2.272)

3.2. Identification of *Gallibacterium* species using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry analysed by multilocus sequence analysis

Manuscript submitted

3.3. MALDI-TOF MS reveals different clonal lineages of Gallibacterium anatis within a defined population

Manuscript in preparation

3.1. Species-specific identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis

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Running title: Identification of food-borne pathogens by MALDI-TOF MS
Key words: MALDI-TOF MS, *Arcobacter*, *Helicobacter*, *Campylobacter*, identification

Summary

Rapid and reliable identification of *Arcobacter* and *Helicobacter* species and their distinction from phenotypically similar *Campylobacter* species has become increasingly important, since many of them are now recognised as human and/or animal pathogens. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to be a rapid and sensitive method for characterisation of microorganisms. In this study, we therefore established a reference database of selected *Arcobacter, Helicobacter* and *Campylobacter* species for MALDI-TOF MS identification. Besides the species with significance as food-borne pathogens - *Arcobacter butzleri*, *Helicobacter pullorum*, *Campylobacter jejuni* and *Campylobacter coli*- several other members of these genera were included in the reference library to determine the species specificity of the designed MALDI Biotyper reference database library. Strains that made up the reference database library were grown on Columbia agar and yielded reproducible and unique mass spectra profiles, which were compared with the Bruker Biotyper database, version 2. The database was used to identify 144 clinical isolates using whole spectral profiles. Furthermore, reproducibility of MALDI-TOF MS results was evaluated in respect to age and/or storage of bacteria and different growth media. It was found that correct identification could be obtained even if the bacteria are stored at room temperature or at + 4°C up to 9 days until being tested. In addition, bacteria were correctly identified when grown on Campylosel-agar, however not when grown on modified charcoal cefoperazone deoxycholate agar. These results indicate that MALDI-TOF MS fingerprinting is a fast and reliable method for the identification of *Arcobacter* and *Helicobacter* species and their distinction from phenotypically similar *Campylobacter* species, with applications in clinical diagnostics.

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Introduction

Identification of species belonging to the *Arcobacter*, *Helicobacter* and *Campylobacter* genera has become increasingly important, since many of them are recognised as human and/or animal pathogens. *Arcobacter butzleri* was found to be the fourth most frequently isolated *Campylobacter*-like organism in human clinical samples, before *C. lari* but after *C. jejuni*, *C. coli*, and *C. fetus* in Belgium and in France (Vandenberg *et al.*, 2004; Prouzet-Mauleon *et al.*, 2006). On several occasions, *Helicobacter pullorum* has been isolated from poultry (Atabay *et al.*, 1998; Neubauer & Hess, 2006b; Zanoni *et al.*, 2007; Ceelen *et al.*, 2007). A number of research groups have associated *H. pullorum* with gastroenteritis, diarrhoea, and liver and gall bladder disease in human patients (Stanley *et al.*, 1994; Young *et al.*, 2000; Rocha *et al.*, 2005; Castera *et al.*, 2006). For *H. pullorum* there is a lack of phenotypic identification methods and as a result, this bacterium is commonly misidentified as thermophilic *Campylobacter* (Atabay *et al.*, 1998; Kuijper *et al.*, 2003). *C. jejuni* is the leading cause of bacterial gastroenteritis in developed countries (EFSA, 2005). In humans the majority (97%) of food-borne diseases can be attributed to animals farmed for meat, especially poultry (Wilson *et al.*, 2008).

Various molecular DNA-based methods for the identification of *Arcobacter, Helicobacter* and *Campylobacter* species have been developed. These methods typically require the use of several species-specific PCR primers, hybridisation probes, or multiple restriction enzymes and are usually not designed to differentiate all known species simultaneously (Bohr *et al.*, 2002; Jauk *et al.*, 2003; Neubauer & Hess, 2006a; Wilson *et al.*, 2008). Bacterial identification by MALDI-TOF MS is based on generating complex fingerprints of biomarker molecules by measuring the exact ratio mass/charge of peptides and proteins (Claydon *et al.*, 1996; Suh & Limbach, 2004b). A number of species from the *Campylobacter* genus (Mandrell *et al.*, 2005; Kolinska *et al.*, 2008) have been characterised by MALDI-TOF MS. In addition, *Helicobacter pylori* and *Helicobacter mustelae* were analysed by MALDI-TOF MS (Winkler *et al.*, 1999), but not *Helicobacter pullorum* and *Helicobacter pamentensis*. So far, no study applying MALDI-TOF MS based on the same sample preparation, technology and using whole spectral profile to differentiate potentially confounding *Arcobacter* and

Helicobacter species together with phenotypically similar *Campylobacter* species is reported.

In this study, we established a reference database of selected *Arcobacter*, *Helicobacter* and *Campylobacter*. The second objective was to use the reference database to identify 144 clinical isolates and compare the results to molecular method. The third objective was to evaluate the reproducibility using different growth media and age of bacteria that are relevant in a diagnostic lab.

Methods

Bacterial strains

For establishing the database library, reference strains were obtained from the National Collection of Type Culture (NCTC), and from the American Typing Culture Collection (ATCC). Preliminary work was done first on *Campylobacter jejuni* NCTC 12744, *Arcobacter butzleri* NCTC 12481 and *Helicobacter pullorum* ATCC 51801 to optimise the method. For standardising the culture method all bacteria were grown on Columbia agar (COS) containing 5 % sheep blood (BioMerieux, Vienna, Austria), at 42°C for 48 hours under microaerobic conditions (GENbox microaer, BioMerieux, Vienna, Austria). In addition, bacteria were grown on Campylosel-Agar (CAM, BioMerieux, Vienna, Austria) and on modified CCD agar (mCCDA, blood-free agar base with cefoperazone 32 mg/l and amphotericin at 10 mg/ml, Oxoid, Cambridge, UK) for reproducibility testing. *Escherichia coli* strain DH5 alpha (Invitrogen) was grown on COS agar at 37 °C for 24 hours.

Reference strains used in this study to generate the database library are listed in Table 1 including their origin, species and other information, used in this study to generate the database library. The reference library was then used to identify 144 clinical isolates that were obtained from humans, environment and from different farm animals but mostly poultry (Table 2).

PCR-RFLP analyses

To identify the clinical isolates by molecular methods, the protocol of Jauk *et al*. (2003) was followed. Briefly, the isolates were examined by polymerase chain reaction-

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restriction fragment-length polymorphism (PCR-RFLP). PCR based on the 16S rRNA gene of the genera *Arcobacter*, *Helicobacter* and *Campylobacter* amplified a 1216-bp fragment. The amplicons were digested with the restriction enzymes *Rsa*I and *Eco*RV. Additional differentiation was obtained using PCR assay based on the hippuricase gene (Marshall *et al.*, 1999).

Table 1. Reference strains used to establish the referenced database for MALDI-TOF MS based species identification.

Designation	Genus	Species	Subspecies	Origin
NCTC 12145	*Campylobacter*	*jejuni*	*jejuni*	human
NCTC 12744	*Campylobacter*	*jejuni*	*jejuni*	contaminated milk
ATCC 700819	*Campylobacter*	*jejuni*	*jejuni*	human faeces
NCTC 12143	*Campylobacter*	*coli*		no information given
NCTC 12144	*Campylobacter*	*lari*		child with mild diarrhoea
NCTC 11458	*Campylobacter*	*lari*		child with mild diarrhoea
ATCC 35217	*Campylobacter*	*hyointestinalis*		swine with proliferative enteritis
NCTC 10842	*Campylobacter*	*fetus*	*fetus*	brain of sheep fetus
NCTC 12481	*Arcobacter*	*butzleri*		human faeces
ATCC 49616	*Arcobacter*	*butzleri*		human faeces
ATCC 49942	*Arcobacter*	*butzleri*		no information given
ATCC 49615	*Arcobacter*	*cryaerophilus*		human blood
ATCC 51400	*Arcobacter*	*skirrowii*		cow (abomasitis)
ATCC 51801	*Helicobacter*	*pullorum*		asymptomatic broiler chicken
ATCC 51802	*Helicobacter*	*pullorum*		human faeces
ATCC 51478	*Helicobacter*	*pamentensis*		tern
DH5 alpha	*Escherichia*	*coli*		

Sample preparation for MALDI-TOF MS analysis

A single colony-forming unit was removed from the agar plates using an inoculating loop and the material was placed in a vial containing 300 µl MilliQ purified water to suspend the bacteria. To inactivate the bacteria, 900 µl of absolute ethanol was added to the vial. After centrifugation for 2 min at 20,000×g the supernatant was removed. Afterwards, a second centrifugation step was done to remove EtOH completely. For cell wall disruption, 50 µl of formic acid (70 %) was added to the pellet and thoroughly mixed. Subsequently, 50 µl acetonitrile were added for protein extraction. After a centrifugation for 3 minutes at 20,000×g, 1 µl of the supernatant containing the bacterial

extract was transferred to a sample position of a ground/polished steel MALDI target plate and allowed to dry at room temperature. Each sample was spotted six times onto the MALDI target plate to test technical replication. Then, the sample was overlaid with 2 µl of matrix (alpha-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluor acetic acid) and dried again. All steps were performed at room temperature.

MALDI-TOF MS parameters

Mass-spectra were collected using Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) in linear mode i.e. using a mass range of 2,000 to 20,000 Dalton (parameter setting: IS1 20.0 kV, IS2 18.7 kV, lens 6.25 kV, detector gain 1634 V). Five hundred single spectra (10 × 50 laser shots) were summarised with a 50 Hz nitrogen laser for each sample. The instrument was externally calibrated with the *Escherichia coli* strain DH5 alpha ribosomal proteins e.g. RL36 4364.3 m/z, RS32 5095.8 m/z, RS34 5380.4 m/z, RS33meth. 6254.4 m/z, RL29 7273.5 m/z, and RS19 10299.1 m/z.

Data visualization and analysis

Each individual spectrum was scrutinised by eye in the flexAnalysis software 3.0 (Bruker Daltonik) and atypical spectra were excluded from further analysis (e.g. flat line spectrum, spectrum containing high matrix background signal). A reference database library was established for MALDI-TOF MS-based species identification following the manufacturer's recommendations for Ultraflex measurement and MALDI Biotyper 1.1 software package (Bruker Daltonik). In brief, for each database entry, at least 30 individually measured mass spectra fingerprints were imported into the MALDI Biotyper 1.1 software. Eight independent measurements (i.e. bacteria were grown at eight different times over the course of three months and subsequently measured) were obtained at six different spots, each. After smoothing, baseline correction, and peak-picking, the resulting peak lists (up to 70 peak masses) were used by the program to calculate and to store a main spectrum containing the information about average peak masses, average peak intensities and peak frequency.

Species identification of clinical isolates by MALDI-TOF MS

The MALDI-TOF MS reference database was used to identify and differentiate 144 clinical isolates. For correct identification of species, a generated peak list was matched

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against the established reference library using the integrated pattern-matching algorithm of MALDI Biotyper 1.1 software. Briefly, the software calculates log(score) values, that is, a log(score) between 1.7 and 2.0 represents genus identification. Log(score) value of ≥ 2.0 represents an identification at species level. Anything less then 1.7 log(score) was rated as not identifiable by the software.

MALDI-TOF MS reproducibility test

To test reproducibility of MALDI-TOF MS-based species identification, 15 field strains were selected randomly and their reproducibility of spectra under different conditions were tested. At first, bacteria were grown on COS agar for 48 hours at 42°C. Subsequently they were stored for 2, 4, 6 and 9 days at room temperature (20°C) and at 4°C to see if age and/or storage conditions of bacteria have an influence on the results. Secondly, these selected strains were grown for 48 hours at 42°C on CAM and mCCD agars that are regularly used to isolate such bacteria.

Results and Discussion

Detection and characterisation of infectious microorganisms in a reasonably fast and reliable manner from biological and environmental samples has a high priority. The genera *Arcobacter, Helicobacter* and *Campylobacter* belong to the rRNA superfamily VI. The differentiation between members of these three genera is challenging, particularly when employing biochemical tests as the sole criterion due to the inconsistency of the phenotypic profiles observed among strains (On, 1996). To overcome the problems related to classical phenotypic species identification methods, this study evaluated the capability of MALDI-TOF MS to differentiate and identify these species. For comparison, PCR-RFLP a classical genotypic method was chosen to characterise clinical strains.

MALDI-TOF MS reference database was established with 16 well-characterised culture collection strains from different sources representing 10 different species of *Arcobacter, Helicobacter* and *Campylobacter*. These species yielded reproducible and unique mass spectral profiles, which were compared with Bruker Biotyper database, version 2. However, Bruker Biotyper database version 2, did not contain spectra from *Helicobacter pamentensis*, as a results this bacteria did not match with any other spectra

in the database. Therefore, *H. pamentensis* mass spectral profile is unique. All reference strains used represented the majority of clinical relevant species from the three genera. In addition, *Escherichia coli* DH5 alpha spectra were included in the reference database library.

The most common *Arcobacter* species were analyzed, namely *A. butzleri*, *Arcobacter skirrowii* and *Arcobacter cryaerophilus* (Figure 1). Interestingly, mass signal patterns of *A. cryaerophilus* and *A. skirrowii* as well as *A. butzleri* and *A. skirrowii* shared a number of common mass peaks that were 100 % frequent (in 135 spectra). Mass signal patterns of *A. cryaerophilus* were completely different to *A. butzleri*. However, the spectra of this *A. cryaerophilus* were found to have a number of small peak-shifts compared to the *A. cryaerophilus* type-strain in Bruker Biotyper database, version 2. As a result, our reference *A. cryaerophilus* strain mass spectra profile did not reliably match the Bruker *A. cryaerophilus* type-strain. This confirms the need to have a number of different strains of the same species coming from different sources in the reference databank for correct identification.

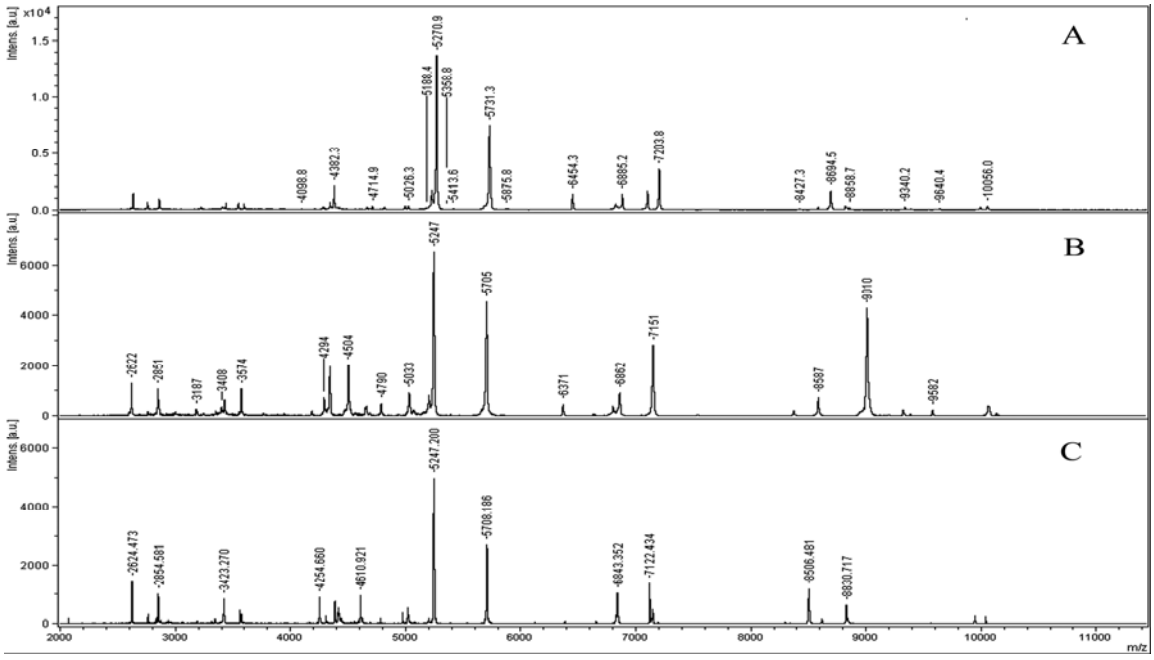

Figure 1. MALDI-TOF mass spectrometric profiles obtained from the analysis of *A. butzleri* (A), *A. skirrowii* (B) and *A. cryaerophilus* (C). The relative intensities of the ions are shown on the y axis, and the masses (in Da) of the ions are shown on the x axis. The m/z value stands for mass to charge ratio. For a

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single positive charge, this value corresponds to the molecular weight of the protein.

Similarly, the two *Helicobacter* species, *Helicobacter pullorum* and *Helicobacter pamentensis* (Figure 2), had very different mass signal patterns and thus can easily be discriminated. Finally, five species from the genus *Campylobacter* widely found as human and/or animal pathogens were analysed, *C. jejuni*, *C. coli, C. lari, Campylobacter hyointestinalis* and *C. fetus*. Representative fingerprint mass spectra clearly demonstrate that all five species produced unique molecular profiles and can be easily differentiated (Figure 3). Within the species fingerprints, it was observed that for thermophilic *Campylobacter* (i.e. *C. jejuni*, *C. coli* and *C. lari*) the mass signal patterns were very different to the non-thermophilic *Campylobacter* (i.e. *C. fetus* and *C. hyointestinalis*). Therefore, the 10 species can be easily discriminated by means of a direct comparison of the whole mass spectra fingerprints.

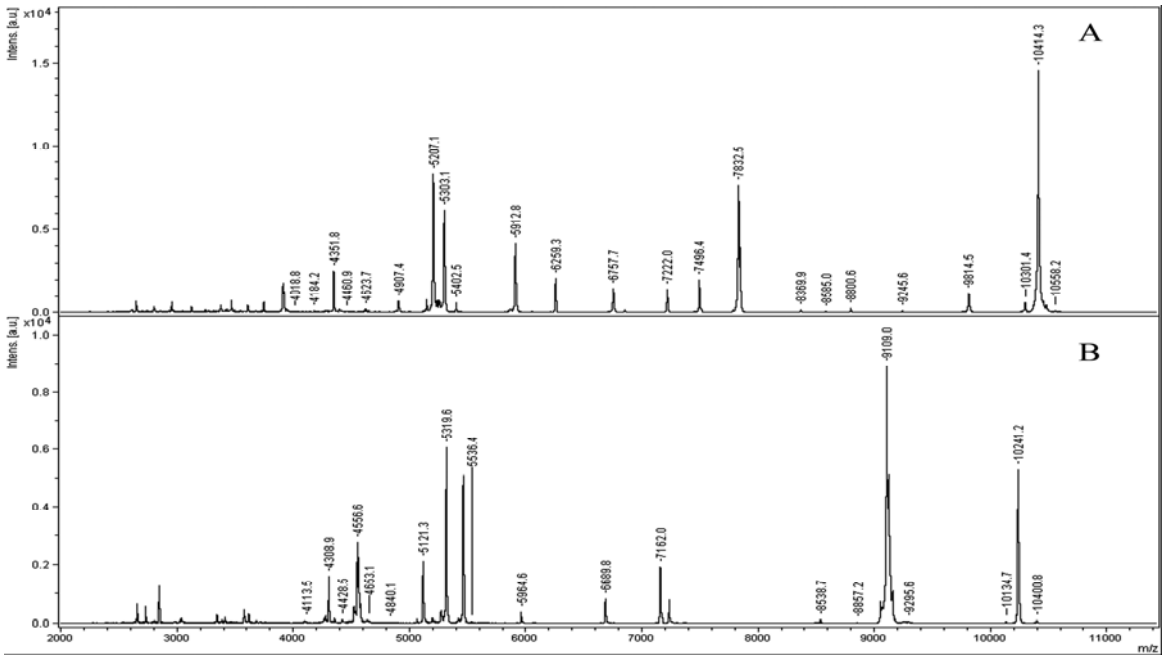

Figure 2. MALDI-TOF mass spectrometric profiles obtained from the analysis of *H. pullorum* (A) and *H. pamentensis* (B). The relative intensities of the ions are shown on the y axis, and the masses (in Da) of the ions are shown on the x axis. The m/z value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein.

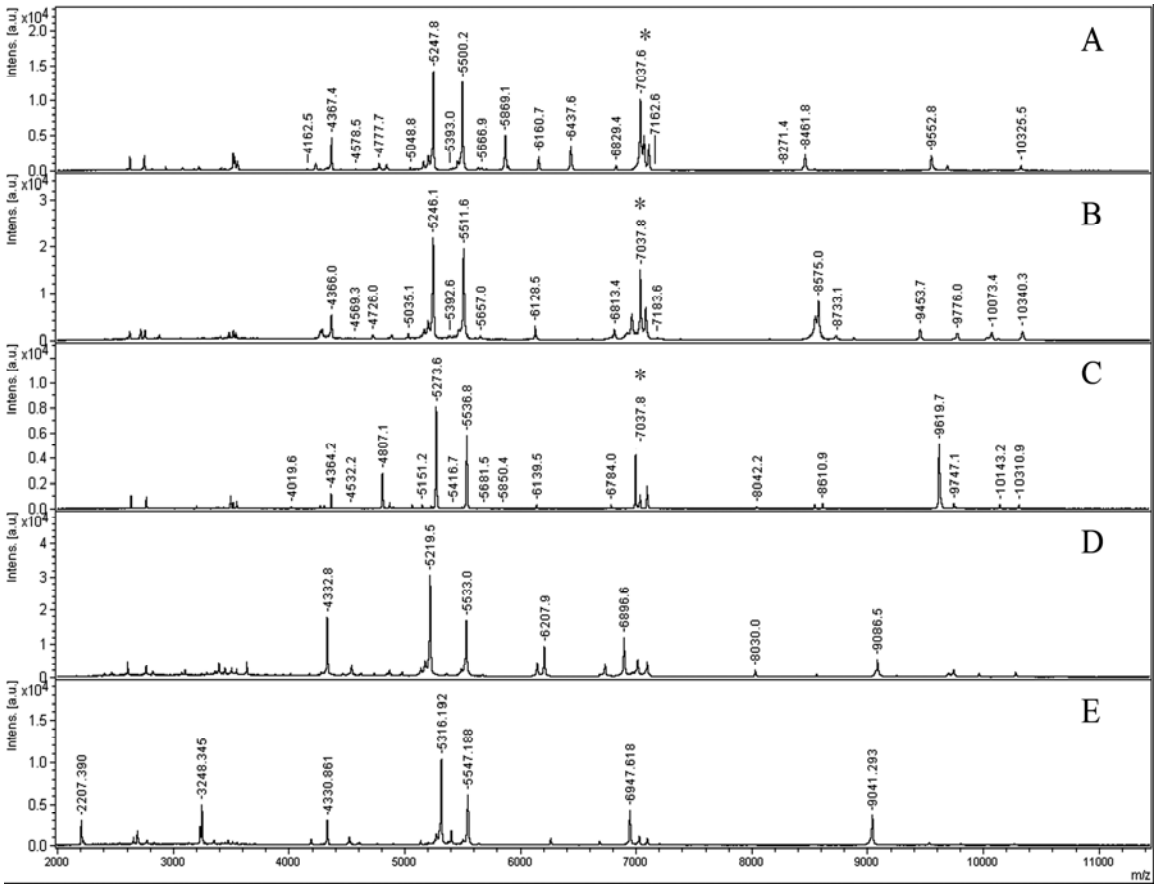

Figure 2. MALDI-TOF mass spectrometric profiles obtained from the analysis of *C. jejuni* (A), *C. coli* (B), *C. lari* (C), *C. fetus* (D) and *C. hyointestinalis* (E). 50S ribosomal L29 protein "biomarker" (*). The relative intensities of the ions are shown on the y axis, and the masses (in Da) of the ions are shown on the x axis. The m/z value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein.

Different mass spectrometric methods for analysing whole bacteria cells for intact proteins including the identification of one or few specific protein biomarker ions, have been reported. Mandrell *et al*. (2005) focused on *Campylobacter* "species-identifying" biomarker ions (SIBS) using MALDI-TOF MS and reported that there were SIBS types associated with source. In addition, biomarker ions in the 9 to 14 kDa range were reported to be diagnostic of *Campylobacter* species. However, one of the biomarkers (7,035 Da see Figure 3 (*)) published by Mandrell *et al.* (2005), was found to be 100 % frequent (in 211 spectra) for thermophilic *Campylobacter* but not observed in non-thermopilic *Campylobacter*, in this study. This biomarker was extracted and identified to be a 50S ribosomal L29 protein by Fagerquist *et al*. (2006). In addition, specific *C.*

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jejuni biomarker 10,276 Da and specific *C. coli* biomarkers 10,032 and 12,855 Da proposed by Mandrell *et al.* (2005) were not frequently detected in this study. Hence, species identification using one or few biomarker ions characteristic for a given species may lead to incorrect results (Kolinska *et al.*, 2008). In another study, *C. jejuni*, *C. coli* and *C. fetus* were compared with *H. pylori* and *H. mustelae* by direct analysis of individual culture colonies in 50 % methanol using MALDI-TOF MS. A few specific biomarkers ions in the 10 to 20 kDa range were reported to be the most discriminative of those observed (Winkler *et al.*, 1999). However, in this study mass signal patterns were only observed in the 2 to 11 kDa range for *Arcobacter*, *Helicobacter* and for *Campylobacter*.

Table 2. Species identification results of the clinical isolates using MALDI-TOF MS in comparison to PCR-RFLP.

Isolation and Identification	**Number of Isolates**					
	A. butzleri	*H. pullorum*	*C. coli*	*C. jejuni*	*C. hyointestinalis*	*C. fetus*
Origin Human	3	8	-	-	-	-
Broiler	-	1	19	62	-	-
Swine	-	-	34	1	-	-
Layer	-	-	3	3	-	-
Bovine	-	-	1	2	2	-
Cat	-	-	-	-	-	1
Duck	-	-	-	1	-	-
Environment	-	-	-	3	-	-
Methods MALDI-TOF MS [a]	3/3	9/9	57/57	72/72	2/2	1/1
PCR-RFLP [b]	3/3	9/9	57/57	72/72	0/2	0/1

Abbreviations: *A.*, *Arcobacter*; *C.*, *Campylobacter*; *H.*, *Helicobacter*

[a] Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry represents an identification at species level log(score) value of ≥ 2.0.

[b] Polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) analyses according to the protocol of Jauk *et al*. (2003).

For evaluation of the method, 144 clinical isolates were used in this study. In parallel to MALDI-TOF MS identification, all strains were analysed by PCR-RFLP. Identification results of the clinical isolates obtained by MALDI-TOF MS and PCR-RFLP are shown in Table 2. List of log(score) is available as supplementary data in JMM Online. All

clinical isolates obtained from humans, environment and from different farm animals, though mostly poultry, gave sufficient spectra for species identification by MALDI-TOF MS. In total, MALDI-TOF MS identified all 144 clinical isolates at species level i.e. log(score) ≥ 2.0 and no differences in results were found associated with the source of the isolate. PCR-RFLP could not differentiate between *Campylobacter fetus* and *Campylobacter hyointestinalis* in three clinical isolates (898, 1147 and 1307). In all these cases, a differentiation at species level with MALDI-TOF MS was possible.

To investigate the stability of clinical isolates identification, 15 different strains were grown according to the standard procedure and subsequently stored at room temperature (20 °C) and at +4 °C and tested after 2, 4, 6 and 9 days. In all cases, mass spectrometry resulted in identical, correct identification results related to the reference database (data not shown). This finding supports the results of a former investigation (Mellmann *et al.*, 2008), where non-fermenting bacteria were stored at room temperature up to 7 days. This can be a great advantage if samples are conducted for a single run or where samples need to be reinvestigated. It is noteworthy, that bacteria grown for 48 hours on COS or CAM agar gave the best results, e.g. good quality spectra, reliable log(score). However, bacteria grown longer than 72 hours gave poor spectra profiles, lower intensity and unspecific peaks. Nevertheless, in most cases correct identification of bacteria could be obtained. In another study MALDI-TOF MS was used for identification of *Listeria*, it was reported that extended periods of growth (4 days tested) did not affect spectra quality and results (Barbuddhe *et al.*, 2008). Obviously, this is species dependent. However, in a separate study *Escherichia coli* was analysed using MALDI-TOF MS, where growth time did not affect the bacterial molecular profile significantly, hence, the incubation time span was up to 48 hours (Mazzeo *et al.*, 2006).

To determine the influence of cultivation media on the quality of spectra, the 15 clinical strains were grown on different types of solid media used to isolate such bacteria. Species identification was possible at the species level if bacteria were grown on CAM agar, which was not used to create the reference database. Interestingly *Arcobacter*, *Helicobacter* and *Campylobacter* grown on mCCD agar could not be used for MALDI-TOF MS species identification as very poor spectra pattern were obtained if any, consequently, no identification was possible (data not shown). Apparently, mCCD agar contaminants interfere with ionisation of biomolecules of the bacteria. In fact, in some

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cases it was not possible to pick up bacteria biomass from mCCD agar because the bacteria were strongly attached on this agars surface. The importance of mCCD agar could not be overlooked as a drawback of MALDI-TOF MS as this agar is commonly used to isolate strains of *Campylobacteraceae*. This means that additional sub-culturing is necessary if the bacteria are going to be used for MALDI-TOF MS identification. To our knowledge, no study published results on using mCCD agar for microorganism identification by MALDI-TOF MS.

Altogether, these data show that MALDI-TOF MS fingerprinting is a fast and reliable method for the identification of *Arcobacter* and *Helicobacter* species and their distinction from phenotypically similar *Campylobacter* species with applications in clinical diagnostics. As a result, considering the speed with which reliable identification can be obtained, this technique is well suited for large-scale research and diagnostic analyses.

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References

1. **Atabay, H. I., Corry, J. E. & On, S. L. (1998).** Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. *J Appl Microbiol* **84,** 1017-1024.

2. **Barbuddhe, S. B., Maier, T., Schwarz, G., Kostrzewa, M., Hof, H., Domann, E., Chakraborty, T. & Hain, T. (2008).** Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* **74,** 5402-5407.

3. **Bohr, U. R. M., Primus, A., Zagoura, A., Glasbrenner, B., Wex, T. & Malfertheiner, P. (2002).** A group-specific PCR assay for the detection of *Helicobacteraceae* in human gut. *Helicobacter* **7,** 378-383.

4. **Castera, L., Pedeboscq, A., Rocha, M. & other authors (2006).** Relationship between the severity of hepatitis C virus-related liver disease and the presence of *Helicobacter* species in the liver: A prospective study. *World J Gastroenterol* **12,** 7278-7284.

5. **Ceelen, L. M., Decostere, A., Chiers, K., Ducatelle, R., Maes, D. & Haesebrouck, F. (2007).** Pathogenesis of *Helicobacter pullorum* infections in broilers. *Int J Food Microbiol* **116,** 207-213.

6. **Claydon, M. A., Davey, S. N., EdwardsJones, V. & Gordon, D. B. (1996).** The rapid identification of intact microorganisms using mass spectrometry. *Nat Biotechnol* **14,** 1584-1586.

7. **EFSA (2005).** Scientific Report of the Scientific Panel on Biological Hazards on the request from the Commission related to *Campylobacter* in animals and foodstuffs., pp. 1-105 Annex to *The EFSA Journal* (2005).

8. **Fagerquist, C. K., Bates, A. H., Heath, S., King, B. C., Garbus, B. R., Harden, L. A. & Miller, W. G. (2006).** Sub-speciating *Campylobacter jejuni* by proteomic analysis of its protein biomarkers and their post-translational modifications. *J Proteome Res* **5,** 2527-2538.

9. **Jauk, V., Neubauer, C., Szolgyenyi, W. & Vasicek, L. (2003).** Phenotypic and genotypic differentiation of *Campylobacter* spp. isolated from Austrian broiler farms: a comparison. *Avian Pathol* **32,** 33-37.

10. **Kolinska, R., Drevinek, M., Jakubu, V. & Zemlickova, H. (2008).** Species identification of *Campylobacter jejuni* ssp. *jejuni* and *C. coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and PCR. *Folia Microbiol (Praha)* **53,** 403-409.

11. **Kuijper, E. J., Stevens, S., Imamura, T., De, W. B. & Claas, E. C. (2003).** Genotypic identification of erythromycin-resistant *campylobacter* isolates as *helicobacter* species and analysis of resistance mechanism. *J Clin Microbiol* **41,** 3732-3736.

12. **Mandrell, R. E., Harden, L. A., Bates, A., Miller, W. G., Haddon, W. F. & Fagerquist, C. K. (2005).** Speciation of *Campylobacter coli*, *C-jejuni*, *C-helveticus*, *C-lari*, *C-sputorum*, and *C-upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* **71,** 6292-6307.

13. **Marshall, S. M., Melito, P. L., Woodward, D. L., Johnson, W. M., Rodgers, F. G. & Mulvey, M. R. (1999).** Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol* **37,** 4158-4160.

Journal of Medical Microbiology (2010), 59, 295-301
DOI 10.1099/jmm.0.016576-0

14. **Mazzeo, M. F., Sorrentino, A., Gaita, M., Cacace, G., Di Stasio, M., Facchiano, A., Comi, G., Malorni, A. & Siciliano, R. A. (2006).** Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the discrimination of food-borne microorganisms. *Appl Environ Microbiol* **72,** 1180-1189.

15. **Mellmann, A., Cloud, J., Maier, T. & other authors (2008).** Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol* **46,** 1946-1954.

16. **Neubauer, C. & Hess, M. (2006a).** Detection and identification of food-borne pathogens of the genera *Campylobacter*, *Arcobacter* and *Helicobacter* by multiplex PCR in poultry and poultry products. *J Vet Med B Infect Dis Vet Public Health* **53,** 376-381.

17. **Neubauer, C. & Hess, M. (2006b).** Tissue tropism of *Helicobacter pullorum* in specific pathogen-free chickens determined by culture and nucleic acid detection. *Avian Dis* **50,** 620-623.

18. **On, S. L. (1996).** Identification methods for *campylobacters*, *helicobacters*, and related organisms. *Clin Microbiol Rev* **9,** 405-422.

19. **Prouzet-Mauleon, V., Labadi, L., Bouges, N., Menard, A. & Megraud, F. (2006).** *Arcobacter butzleri*: Underestimated enteropathogen. *Emerg Infect Dis* **12,** 307-309.

20. **Rocha, M., Avenaud, P., Menard, A., Le, B. B., Balabaud, C., Bioulac-Sage, P., de Magalhaes Queiroz, D. M. & Megraud, F. (2005).** Association of *Helicobacter* species with hepatitis C cirrhosis with or without hepatocellular carcinoma. *Gut* **54,** 396-401.

21. **Stanley, J., Linton, D., Burnens, A. P., Dewhirst, F. E., On, S. L. W., Porter, A., Owen, R. J. & Costas, M. (1994).** *Helicobacter Pullorum* Sp-Nov - Genotype and Phenotype of A New Species Isolated from Poultry and from Human Patients with Gastroenteritis. *Microbiol* **140,** 3441-3449.

22. **Suh, M. J. & Limbach, P. A. (2004).** Investigation of methods suitable for the matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins from ribonucleoprotein complexes. *Eur J Mass Spectrom (Chichester, Eng)* **10,** 89-99.

23. **Vandenberg, O., Dediste, A., Houf, K. & other authors (2004).** *Arcobacter* species in humans. *Emerg Infect Dis* **10,** 1863-1867.

24. **Wilson, D. J., Gabriel, E., Leatherbarrow, A. J. & other authors (2008).** Tracing the source of *campylobacteriosis*. *PLoS Genet* **4,** e1000203.

25. **Winkler, M. A., Uher, J. & Cepa, S. (1999).** Direct analysis and identification of *Helicobacter* and *Campylobacter* species by MALDI-TOF mass spectrometry. *Anal Chem* **71,** 3416-3419.

26. **Young, V. B., Chien, C. C., Knox, K. A., Taylor, N. S., Schauer, D. B. & Fox, J. G. (2000).** Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. *J Infect Dis* **182,** 620-623.

27. **Zanoni, R. G., Rossi, M., Giacomucci, D., Sanguinetti, V. & Manfreda, G. (2007).** Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. *Int J Food Microbiol* **116,** 168-173.

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Supplementary Table S1. Identification results of the 144 clinical isolates using MALDI-TOF MS in comparison to PCR-RFLP.

Isolate No.	Origin	PCR-RFLP [a]		MALDI-TOF MS	
		Species ID	Level of ID	Species ID	Log(score) [b]
B01-33	Human faecal sample	*A. butzleri*	Species	*A. butzleri*	2.440
B01-404	Human faecal sample	*A. butzleri*	Species	*A. butzleri*	2.548
B01-66	Human faecal sample	*A. butzleri*	Species	*A. butzleri*	2.538
CS03-13	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.658
CS03-14	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.580
CS03-15	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.566
CS03-3	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.549
CS03-7	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.650
CS03-8	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.567
H01-605	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.622
08-89916-2	Human faecal sample	*H. pullorum*	Species	*H. pullorum*	2.622
08-212	Broiler faecal sample	*H. pullorum*	Species	*H. pullorum*	2.657
37	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.582
273	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.579
274	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.613
299	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.466
324	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.376
325	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.600
413	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.308
542	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.197
775	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.593
858	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.629
904	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.558
969	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.445
970	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.515
1031	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.589
1040	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.533
04-2965	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.615
04-1007	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.486
04-1198	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.629
04-1009	Broiler faecal sample	*C. coli*	Species	*C. coli*	2.649
1	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.152
2	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.386
4	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.425
95	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.436
98-100	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.402
98-101	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.443
181	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.592
239	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.629

281	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.503
283	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.529
295	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.603
296	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.487
309	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.479
312	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.518
315	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.403
320	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.377
322	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.361
323	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.287
326	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.581
336	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.554
409	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.564
415	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.565
433	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.566
479	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.364
572	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.464
590	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.503
598	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.525
606	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.527
698	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.473
700	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.432
834	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.552
835	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.567
878	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.602
887	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.618
935	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.525
936	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.499
958	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.546
1007	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.516
1050	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.360
1293	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.407
1294	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.425
1298	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.526
1084	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.459
1240	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.516
1306	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.517
04-1195	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.478
04-1562	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.618
04-1768	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.504
04-1773	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.651
04-1876	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.508
04-1945	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.582
04-2004	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.648

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04-2321	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.566
04-2322	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.549
04-2333	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.603
04-2339	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.623
04-2529	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.511
04-2532	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.626
04-2833	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.522
04-2963	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.638
04-2966	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.659
04-2970	Broiler faecal sample	*C. jejuni*	Species	*C. jejuni*	2.512
346	Swine faecal sample	*C. coli*	Species	*C. coli*	2.550
405	Swine faecal sample	*C. coli*	Species	*C. coli*	2.418
422	Swine faecal sample	*C. coli*	Species	*C. coli*	2.647
492	Swine faecal sample	*C. coli*	Species	*C. coli*	2.427
531	Swine faecal sample	*C. coli*	Species	*C. coli*	2.479
561	Swine faecal sample	*C. coli*	Species	*C. coli*	2.429
614	Swine faecal sample	*C. coli*	Species	*C. coli*	2.595
723	Swine faecal sample	*C. coli*	Species	*C. coli*	2.588
737	Swine faecal sample	*C. coli*	Species	*C. coli*	2.608
774	Swine faecal sample	*C. coli*	Species	*C. coli*	2.580
804	Swine faecal sample	*C. coli*	Species	*C. coli*	2.593
817	Swine faecal sample	*C. coli*	Species	*C. coli*	2.615
877	Swine faecal sample	*C. coli*	Species	*C. coli*	2.628
914	Swine faecal sample	*C. coli*	Species	*C. coli*	2.598
963	Swine faecal sample	*C. coli*	Species	*C. coli*	2.463
975	Swine faecal sample	*C. coli*	Species	*C. coli*	2.542
993	Swine faecal sample	*C. coli*	Species	*C. coli*	2.490
1009	Swine faecal sample	*C. coli*	Species	*C. coli*	2.559
924	Swine faecal sample	*C. coli*	Species	*C. coli*	2.525
1047	Swine faecal sample	*C. coli*	Species	*C. coli*	2.484
1071	Swine faecal sample	*C. coli*	Species	*C. coli*	2.410
1078	Swine faecal sample	*C. coli*	Species	*C. coli*	2.388
1104	Swine faecal sample	*C. coli*	Species	*C. coli*	2.489
1121	Swine faecal sample	*C. coli*	Species	*C. coli*	2.536
1140	Swine faecal sample	*C. coli*	Species	*C. coli*	2.620
1148	Swine faecal sample	*C. coli*	Species	*C. coli*	2.597
1169	Swine faecal sample	*C. coli*	Species	*C. coli*	2.489
1189	Swine faecal sample	*C. coli*	Species	*C. coli*	2.499
1197	Swine faecal sample	*C. coli*	Species	*C. coli*	2.508
1230	Swine faecal sample	*C. coli*	Species	*C. coli*	2.430
1243	Swine faecal sample	*C. coli*	Species	*C. coli*	2.510
1256	Swine faecal sample	*C. coli*	Species	*C. coli*	2.508
1299	Swine faecal sample	*C. coli*	Species	*C. coli*	2.520
1318	Swine faecal sample	*C. coli*	Species	*C. coli*	2.520

836	Swine faecal sample	*C. jejuni*	Species	*C. jejuni*	2.518
829	Layer faecal sample	*C. coli*	Species	*C. coli*	2.633
876	Layer faecal sample	*C. coli*	Species	*C. coli*	2.541
1242	Layer faecal sample	*C. coli*	Species	*C. coli*	2.486
438	Layer faecal sample	*C. jejuni*	Species	*C. jejuni*	2.617
560	Layer faecal sample	*C. jejuni*	Species	*C. jejuni*	2.357
576	Layer faecal sample	*C. jejuni*	Species	*C. jejuni*	2.474
512	Bovine faecal sample	*C. coli*	Species	*C. coli*	2.302
1147	Bovine faecal sample	*C. fetus* or *hyointestinalis*	2 different species possible	*C. hyointestinalis*	2.446
1307	Bovine faecal sample	*C. fetus* or *hyointestinalis*	2 different species possible	*C. hyointestinalis*	2.307
1077	Bovine faecal sample	*C. jejuni*	Species	*C. jejuni*	2.393
1207	Bovine faecal sample	*C. jejuni*	Species	*C. jejuni*	2.281
898	Cat faecal sample	*C. fetus* or *hyointestinalis*	2 different species possible	*C. fetus*	2.497
1244	Duck faecal sample	*C. jejuni*	Species	*C. jejuni*	2.678
1303	Environmental swab	*C. jejuni*	Species	*C. jejuni*	2.417
1304	Environmental swab	*C. jejuni*	Species	*C. jejuni*	2.523
485	Environmental swab	*C. jejuni*	Species	*C. jejuni*	2.361

Abbreviations: *A.*, *Arcobacter*; *C.*, *Campylobacter*; *H.*, *Helicobacter*; ID, identification

[a] Polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) analyses according to the protocol of Jauk *et al*. (2003) .

[b] MALDI Biotyper 1.1 results stand for: species level identification with log(score) values ≥ 2.0 and genus identification with log(score) values between 1.7 and 2.0.

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3.2. Identification of *Gallibacterium* species by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry evaluated by multilocus sequence analysis

Manuscript submitted for publication

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Running title: Identification of *Gallibacterium* species by MALDI

Key words: *Gallibacterium*, MALDI-TOF MS, MLSA, characterization

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) whole cell fingerprinting was used for characterization of 66 reference strains of *Gallibacterium*. The four recognised *Gallibacterium* species and *Gallibacterium* genomospecies 1 yielded reproducible and unique mass spectrum profiles, which were confirmed with Bruker Biotyper reference database version 3. The reproducibility of MALDI-TOF MS results were evaluated varying the age and storage of the cultures investigated. Reliable species identification was possible for up to 8 days of storage at 4°C and less reliable if the bacteria were stored at room temperature (20°C). However, if the strains were grown longer than 48 hrs at 37 °C under microaerobic atmosphere, poor identification results were obtained, due to changes in protein profile. The MALDI-TOF MS results of all 66 strains demonstrated 87.9 % concordance with results based upon biochemical/physiological characterization. In addition, diversities outlined by MALDI-TOF MS were verified by sequencing the *rpoB* (n = 43), 16S rRNA (n = 28), *infB* (n = 14) and *recN* (n = 14) genes (multilocus sequence analysis, MLSA). Furthermore, discrepancies were observed between some of the genes sequenced. Results obtained demonstrated that MALDI-TOF MS fingerprinting represents a fast and reliable method for identification and differentiation of the four recognised *Gallibacterium* species and possible fifth species *Gallibacterium* genomospecies 1, with applications in clinical diagnostics.

Introduction

Four species of *Gallibacterium*, *G. anatis*, *G. melopsittaci*, *G. salpingitidis*, and *G. trehalosifermentans* have been recognized so far (Euzeby, 1997; Christensen *et al.*, 2003; Bisgaard *et al.*, 2009). However, 16S rRNA gene sequence data clearly indicated the existence of a probable new species *G.* genomospecies 1 and *G.* genomospecies 2 (Christensen *et al.*, 2003). In addition, Bisgaard *et al*. (2009) showed that 16S rRNA groups III (*G.* genomospecies 3) and V (*G.* group V) should be classified as novel species of *Gallibacterium*. All taxa of *Gallibacterium* reported so far seem to be associated with birds, although isolates have been reported from cattle and pigs too (Gerlach, 1977; Mushin *et al.*, 1980; Bisgaard & Dam, 1981; Christensen *et al.*, 2003; Jordan *et al.*, 2005; Bisgaard *et al.*, 2009). *G. anatis* is a common organism of the upper respiratory and lower genital tract of poultry. Disease associated with this

microorganism is related to egg peritonitis, decrease in egg production and occasionally an increase in mortality (Gerlach, 1977; Mushin *et al.*, 1980; Bisgaard & Dam, 1981; Jordan *et al.*, 2005; Neubauer *et al.*, 2009). A novel RTX-toxin, GtxA, in *G. anatis* was recently demonstrated as an important virulence factor for haemolytic and leukotoxic activity (Kristensen *et al.*, 2010).

Like, most other genera of the family *Pasteurellaceae* Pohl 1981, the genus *Gallibacterium* represents a phenotypically heterogeneous group (Christensen *et al.*, 2003). Phenotypic characterization therefore constitutes a laborious and time-consuming diagnostic method, which may also give ambiguous results due to variable outcome of tests included. For the same reason interpretation of earlier studies, in which only relatively few phenotypic characters have been investigated might be difficult (Bisgaard, 1993).

Various genotypic methods have been developed for identification of *Gallibacterium*, (Bojesen *et al.*, 2003b; Christensen *et al.*, 2004; Bojesen *et al.*, 2007). The specificity of these methods, however, remains to be investigated including the recently published taxa of *Gallibacterium* (Bisgaard *et al.*, 2009).

Bacterial identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is based on generating complex fingerprint spectra of biomarker molecules by measuring the exact size of peptides and proteins, which are assumed to represent high-abundant proteins with house-keeping functions, such as ribosomal or nucleic-acid binding proteins (Claydon *et al.*, 1996; Suh & Limbach, 2004). The procedure is fast, requires minimal amounts of colony material, is suitable for high-throughput routine analysis, and therefore has a great potential for application in routine clinical microbiology laboratories as reviewed by Carbonnelle *et al*. (Carbonnelle *et al.*, 2010).

In this study, the MALDI Biotyper system was assessed for the first time for identification of taxa of *Gallibacterium*, resulting in a reference database. To achieve this task a well defined population of *Gallibacterium* species was needed. Therefore, from all strains investigated biochemical/physiological characters were obtained. Also, the few already known reference/type strains from different *Gallibacterium* species

were tested. Moreover, the reproducibility of MALDI results obtained was evaluated varying the age and storage conditions of the cultures investigated, which is relevant in a diagnostic laboratory. Since taxonomic investigations have demonstrated difficulties as to obtain correct identification within this area diversities outlined by MALDI-TOF MS were verified by sequencing the rpoB, 16S rRNA, *infB, recN* genes (multilocus sequence analysis, MLSA).

Materials and Methods

Bacterial strains and phenotypic characterization

Sixty-six *Gallibacterium* reference strains were included in this study (Table 1). These strains have previously been characterized in detail by phenotypic methods and a number of these strains have also been characterized by genotypic methods as shown in Table 1 (Christensen *et al.*, 2003; Bojesen *et al.*, 2003a; Bojesen *et al.*, 2003b; Bojesen *et al.*, 2007; Bisgaard *et al.*, 2009). *Pasteurella multocida* 08/14290 field strain was identified by phenotypic methods and Biotyper reference database library version 3. All bacteria were grown on Columbia agar (COS) containing 5 % sheep blood (BioMerieux, Vienna, Austria) and inoculated at 37°C for 24 hours under microaerophilic conditions.

Sample preparation

Sample preparation for MALDI-TOF MS was performed as previously described in detail (Alispahic *et al.*, 2010). Each sample was spotted eight times onto the MALDI target plate to test technical replication. Then, the sample was overlaid with 2 µl of matrix (alpha-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluor acetic acid, according to the protocol of Bruker) and dried again. All steps were performed at room temperature.

MALDI-TOF MS parameters

Mass-spectra were collected using Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) in linear mode i.e. using a mass range of 2,000 to 20,000 Dalton (parameter setting: IS1 20.0 kV, IS2 18.7 kV, lens 6.25 kV, detector gain 1634 V). Five hundred single spectra (10 times 50 shots with a 50 Hz nitrogen laser from different positions of the target spot) were summarised and each

spot was measured 3 times automatically. The instrument was externally calibrated with Bruker bacterial test standard (BTS, Bruker), proteins used for calibration are as follows: ribosomal proteins RL36, 4364.3 m/z; RS32, 5095.8 m/z; RS34, 5380.4 m/z; RS33meth, 6254.4 m/z; RL29, 7273.5 m/z; RS19, 10299.1 m/z; RNase A, 13683.2 m/z and myoglobin, 16952.3 m/z.

Creation of reference database library

Each individual spectrum was scrutinised by eye in the flexAnalysis software 3.0 (Bruker Daltonik GmbH, Leipzig, Germany) and atypical spectra were excluded from further analysis (e.g. flat line spectrum, spectrum containing high matrix background signal). A reference database library was established for MALDI-TOF MS-based species identification following the manufacturer's recommendations for Ultraflex measurement and MALDI Biotyper 2 software package (Bruker Daltonik GmbH, Leipzig, Germany). In brief, for each database entry, at least 20 individually measured mass spectra fingerprints were imported into the MALDI Biotyper 2 software. After smoothing, baseline correction, and peak-picking, the resulting peak lists (up to 70 peak masses) were used by the program to calculate and to store a main spectrum containing the information about average peak masses, average peak intensities and peak frequency.

Dendrogram construction

For strain identification, the formation of the dendrogram is based on cross-wise minimum spanning tree (MSP) matching. Similar MSPs result in a high matching score value. Each MSP is compared with all MSPs of the analysed set. The list of score values is used to calculate normalised distance values between the analysed species, resulting in a matrix of matching scores. The visualization of the respective relationship between the MSPs is displayed in a dendrogram using the standard settings of the MALDI Biotyper 2.0 software. Species with distance levels less than 500 have been described as reliably classified (Sauer *et al.*, 2008). *Pasteurella multocida* strain 08/14290 was used as an out-group in the dendrogram.

MALDI-TOF MS reproducibility test

To test the reproducibility of MALDI-TOF MS-based species identification, eight reference strains were selected randomly and their reproducibility of spectra under five

different conditions were tested. Condition number 1: bacteria were grown on COS agar and incubated for 3 and 8 days at 37 °C under microaerobic conditions. Condition number 2: bacteria were grown on COS agar at 37°C for 24 hours under microaerobic conditions and then left at room temperature (~20°C) under a microaerobic atmosphere. Condition number 3: the same as condition number 2, but incubated and stored in an aerobic atmosphere. Condition number 4: the same as condition number 2 but stored at 4°C. Condition number 5: the same as condition number 3 but with storage at 4°C. For all conditions, a small amount of biomass was used to measure 5 spots (resulting in 5 spectra for each sample) with MALDI-TOF MS after 3 and 8 days, respectively. Resulting spectra were imported into Biotyper software for identification using the already made MSP library used for dendrogram creation.

Sequencing of *rpoB, recN, infB* and 16S rRNA genes

The partial *rpoB* sequence (Table 1) was determined according to Mollet *et al*. (1997) covering the region 509-680 (*Escherichia coli* pos.) of the deduced protein sequence as reported previously (Angen *et al.*, 2003; Korczak *et al.*, 2004). Partial *recN* gene sequences (Table 1) were determined as described by Kuhnert & Korczak (2006), with 1340 bp of the gene being sequenced. Sequencing of *infB* gene (Table 1) was performed according to previously described protocols (Korczak *et al.*, 2004; Kuhnert *et al.*, 2004). Sequencing of the 16S rRNA gene (Table 1) was also performed according to previous reports (Christensen *et al.*, 2002; Kuhnert *et al.*, 2002; Angen *et al.*, 2003). 16S rRNA, *rpoB, recN* and *infB* gene sequences determined in the present investigation have been deposited with GenBank/EMBL/DDBJ under the accession numbers listed in Table 1. Pairwise comparisons for similarity were performed by the program WATER included in EMBOSS (Rice *et al.*, 2000). Multiple alignment and phylogenetic trees were constructed by the neighbour joining method based on Jukes and Cantor corrected similarity matrices by ClustalX (Thompson *et al.*, 1997) and drawn by MEGA4 (Tamura *et al.*, 2007).

Results

A reproducible signal pattern was obtained from all 66 reference *Gallibacterium* strains used for MALDI/Biotyper reference database development (Table 1). Signal patterns obtained were compared with data in the Bruker Biotyper reference database version 3 (contained 3,024 bacterial strains). The Bruker database only contained the type strain of *Gallibacterium anatis* (DSM 16844^T) which matched to the *G. anatis* type strain included in the present study with a 2.703 log(score) value. The four recognised *Gallibacterium* species all yielded unique mass spectral profiles (Figure 1), therefore could be easily differentiated by score values and dendrogram analysis that showed separate clades/clusters for each species in the Biotyper software.

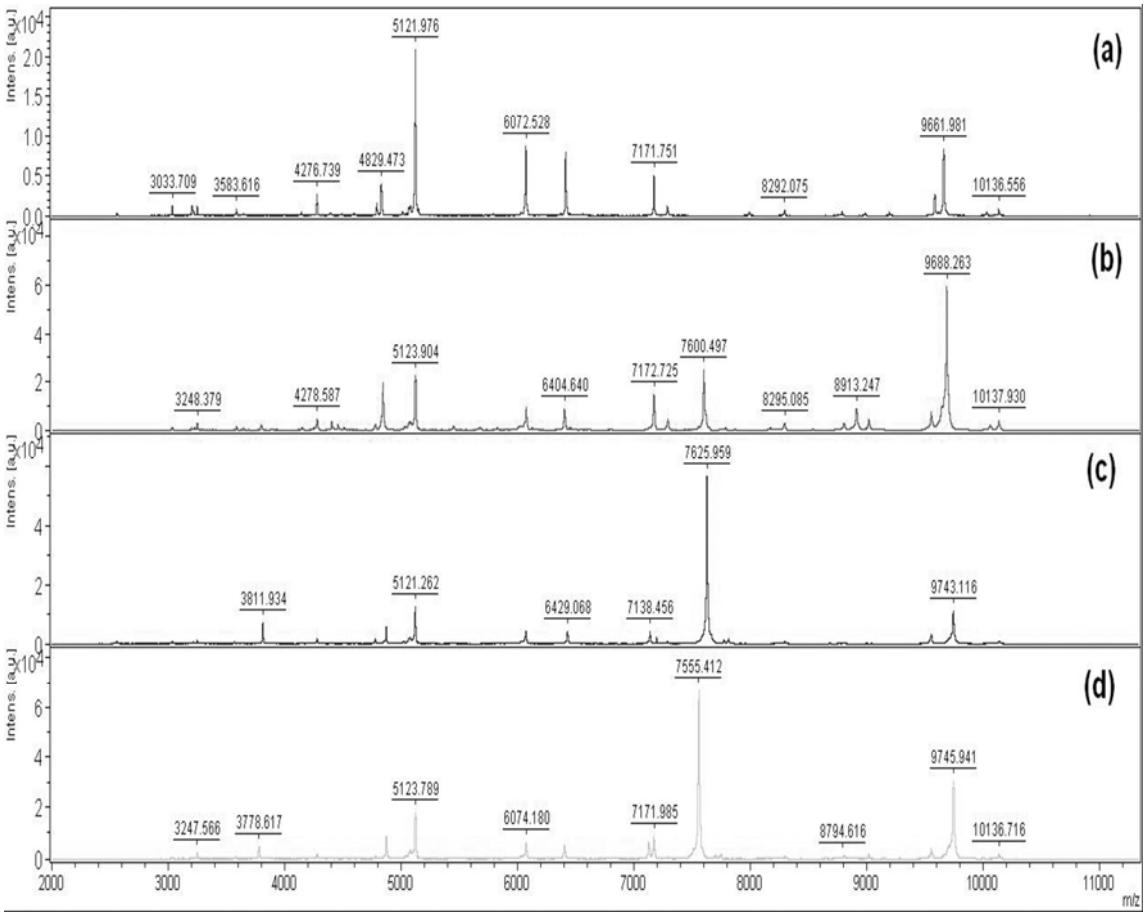

Figure 1.

MALDI-TOF mass spectrometric profiles obtained from the analysis of *Gallibacterium* type strains of species: (A) *G. anatis*, (B) *G. melopsittaci*, (C) *G. salpingitidis* and (D) *G. trehalosifermentans*. The relative intensities of the ions are shown on the y axis, and the masses (in Da) of the ions are shown on the x

axis. The m/z value stands for mass to charge ratio. For a single positive charge, this value corresponds to the molecular weight of the protein.

According to the dendrogram generated by the MALDI Biotyper software based upon mass signals and intensities (Figure 2), the type species of the genus *Gallibacterium*, *G. anatis* (F149^T), clustered with 55 other isolates (cluster 1). This cluster, however, splits into two groups at a distance level between 200 and 300, the majority of isolates including the type strain of the type species clustering below a 200-distance level indicating a high similarity between the isolates. *Gallibacterium* group V (39199/1L) and strain CCM5976 (*Gallibacterium* genomospecies 2) formed a separate cluster with strain P. sp. 38 which branched deeply with the type species of the genus *Gallibacterium* but below the 200 distance-level. Strains 29934liver, CCM5974 (*Gallibacterium* genomospecies 1), 2737/89, CCM5975, 10816/12 and 139/89 formed cluster 2 demonstrating a distance level closer to *G. anatis* (600-500). Moreover, the type strain of *G. salpingitidis* (F150^T) clustered with *G.* genomospecies 3 strain (F151) (cluster 3) at a distance between 200 and 300. Both isolates, however, clustered with the type strain of the type species, *G. anatis*, at a distance above 600. The mass signal pattern of *G. trehalosifermentans* (52/33/90^T) (cluster 4) and of *G. melopsittaci* (F450^T) (cluster 5) differed significantly from *G. anatis* demonstrating a distance level above 800 and differed to each other at a distance level between 800 and 700. The strain relatedness documented in the dendrogram complements identification by score values (data not shown).

Reproducibility was carried out to investigate if identification of eight randomly chosen strains was possible under different growth and storage conditions relevant in a clinical laboratory. Reliable species identification (log(score) above 2.3) was possible after 3 and 8 days of storage at 4°C. Less reliable species identification (6/8 strains correctly identified but with a lower log(score)) was seen if the bacteria were stored at room temperature (~20°C) for 3 days. The reliability declined further after 8 days of storage (3/8 strains correctly identified to species level with log(score) just above 2.0) (data not shown). However, if the strains were grown for 3 or 8 days at 37 °C under microaerobic atmosphere poor identification results were obtained (2/8 strains with log(score) just above 2.0). No distinction was seen if the strains were stored at different atmospheres (data not shown).

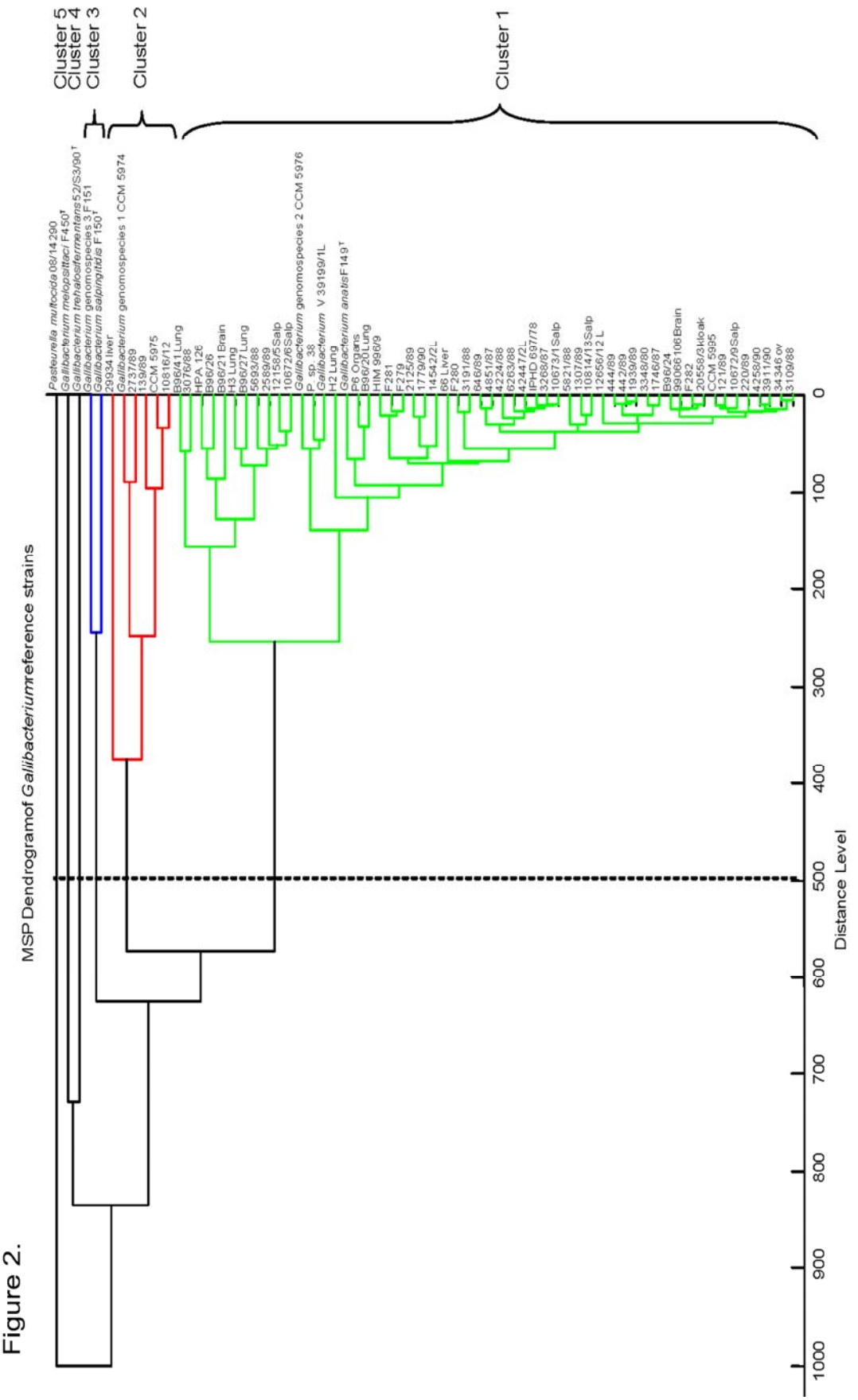
Cluster 5
Cluster 4
Cluster 3
Cluster 2
Cluster 1
Pasteurella multocida 08/14290
Gallibacterium melopsittaci F450T
Gallibacterium trehalosifermentans 52/S3/90T
Gallibacterium genomospecies 3 F151
Gallibacterium salpingitidis F150T
29934 liver
Gallibacterium genomospecies 1 CCM 5974
273/89
139/89
CCM 5975
10816/12
B96/41 Lung
3076/88
HPA 126
B96/26
B96/21 Brain
H3 Lung
B96/27 Lung
5693/88
2589/88
12156/5Salp
10672/6Salp
Gallibacterium genomospecies 2 CCM 5976
P. sp. 38
Gallibacterium V 39199/1L
H2 Lung
Gallibacterium anatis F149T
P6 Organs
B96/20 Lung
HIM 996/9
F281
F279
2125/89
1779/90
14542/2L
66 Liver
F280
3191/88
646/89
4851/87
4224/88
6263/88
4244/12L
IPHD 697/78
3268/87
10673/1Salp
5821/88
1307/89
10814/13Salp
12656/12 L
1444/89
1442/89
1193/89
3348/80
1746/87
B96/24
99/066 106 Brain
F282
20558/3Kloak
CCM 5995
121/89
10672/9Salp
220/89
14258/90
3911/90
34346 ov
1310/88
0
100
200
300
400
500
600
700
800
900
1000
Distance Level
MSP Dendrogram of Gallibacterium reference strains

Figure 2.

Figure 2.

Classification of *Gallibacterium* reference strains investigated. Score–oriented (MSP) dendrogram of MALDI-TOF mass spectral profiles generated by the MALDI Biotyper 2. The dendrogram was generated with following settings: distance measure was set at correlation, linkage at average and score threshold value for a single organism at 600. Strains clustering with distance levels lower than 500 could be classified up to species level.

The strain relatedness documented in the MALDI dendrogram classified 58 strains out of 66 investigated (87.9%) concordant to results based upon biochemical/physiological characterization. Discrepancies were seen with strains 2737/89, 10816/12, CCM5974, CCM5975, 29934 Liver, 139/89, F151 and 39199/1L.

To investigate if clusters outlined by MALDI-TOF MS (Figure 2) also reflect genotypic relationship, the partial *rpoB* sequences of 36 strains were generated and compared with seven strains from GenBank covering the diversity outlined in Figure 2. In general, 30 strains out of 43 were assigned to *G. anatis* with *rpoB* sequence which was concordant to MALDI-TOF MS results, except for strains 10816/12 and 2737/89. Hence, clustering of genomospecies 2 with *G. anatis* was supported by *rpoB* (Figure 3 a). Sequencing of *rpoB* also classified strains CCM5975, 29934 Liver, 139/89 and CCM5974 with *Gallibacterium* genomospecies 1 (Figure 3 a) concordant to MALDI-TOF MS (cluster 2 in Figure 2). Moreover, the clustering of *G. salpingitidis* with genomospecies 3 (cluster 3) was confirmed by *rpoB*. Interestingly, *G. melopsittaci* and *G. trehalosifermentans* clustered together in *rpoB* analysis, while major differences were observed as to *Gallibacterium* group V. However, MALDI-TOF MS clearly identified *G. melopsittaci* and *G. trehalosifermentans* as two different species, though *Gallibacterium* group V was identified as *G. anatis*.

In addition, to verify the diversities in Figure 2 and Figure 3 a, the partial 16S rRNA sequence of 6 strains were generated and compared with 22 strains from GenBank. The *recN* and *infB* sequence of 8 strains were generated and compared with 6 strains (for both genes) from GenBank. Two bovine isolates (B96/20 and B96/27) were classified with *G. salpingitidis*, while a third isolate, B96/41, clustered with *Gallibacterium*

genomospecies 3 with *rpoB* analysis. However, all three isolates identified as *G. anatis* by MALDI-TOF MS, biochemical/physiological characterization, and sequencing of 16S rRNA, *recN* and *infB* genes. Moreover, sequencing of *recN* and *infB* did not allow separation of genomospecies 1 (CCM5974) from *G. anatis* (Figure 3 c-d). Contrary to MALDI-TOF MS, strains 10816/12 and 2737/89 (cluster 2), clustered with *G. anatis* type strain in all genes sequenced (Figure 3 a-d). A concatenated analysis of 16S rRNA, *rpoB*, *recN* and *infB* gene sequences confirmed the existence of the taxa previously outlined by Bisgaard *et al.* (2009) (data not shown).

Fig. 3a. rpoB

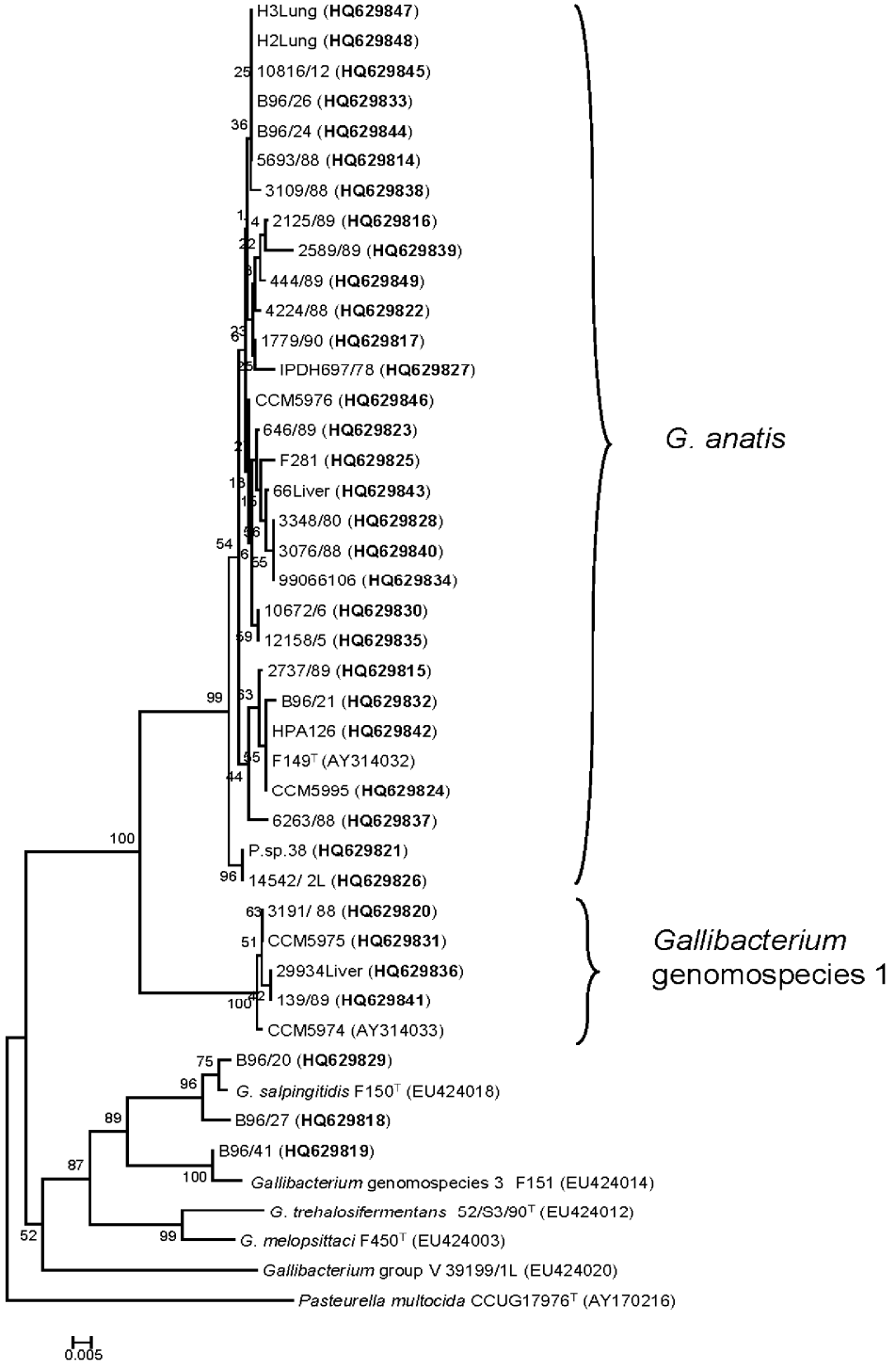
H3Lung (HQ629847)
H2Lung (HQ629848)
10816/12 (HQ629845)
B96/26 (HQ629833)
B96/24 (HQ629844)
5693/88 (HQ629814)
3109/88 (HQ629838)
2125/89 (HQ629816)
2589/89 (HQ629839)
444/89 (HQ629849)
4224/88 (HQ629822)
1779/90 (HQ629817)
IPDH697/78 (HQ629827)
CCM5976 (HQ629846)
646/89 (HQ629823)
F281 (HQ629825)
66Liver (HQ629843)
3348/80 (HQ629828)
3076/88 (HQ629840)
99066106 (HQ629834)
10672/6 (HQ629830)
12158/5 (HQ629835)
2737/89 (HQ629815)
B96/21 (HQ629832)
HPA126 (HQ629842)
F149T (AY314032)
CCM5995 (HQ629824)
6263/88 (HQ629837)
P.sp.38 (HQ629821)
14542/ 2L (HQ629826)
3191/ 88 (HQ629820)
CCM5975 (HQ629831)
29934Liver (HQ629836)
139/89 (HQ629841)
CCM5974 (AY314033)
G. anatis
Gallibacterium genomospecies 1
B96/20 (HQ629829)
G. salpingitidis F150T (EU424018)
B96/27 (HQ629818)
B96/41 (HQ629819)
Gallibacterium genomospecies 3 F151 (EU424014)
G. trehalosifermentans 52/S3/90T (EU424012)
G. melopsittaci F450T (EU424003)
Gallibacterium group V 39199/1L (EU424020)
Pasteurella multocida CCUG17976T (AY170216)
0.005

Fig. 3b. 16S rRNA

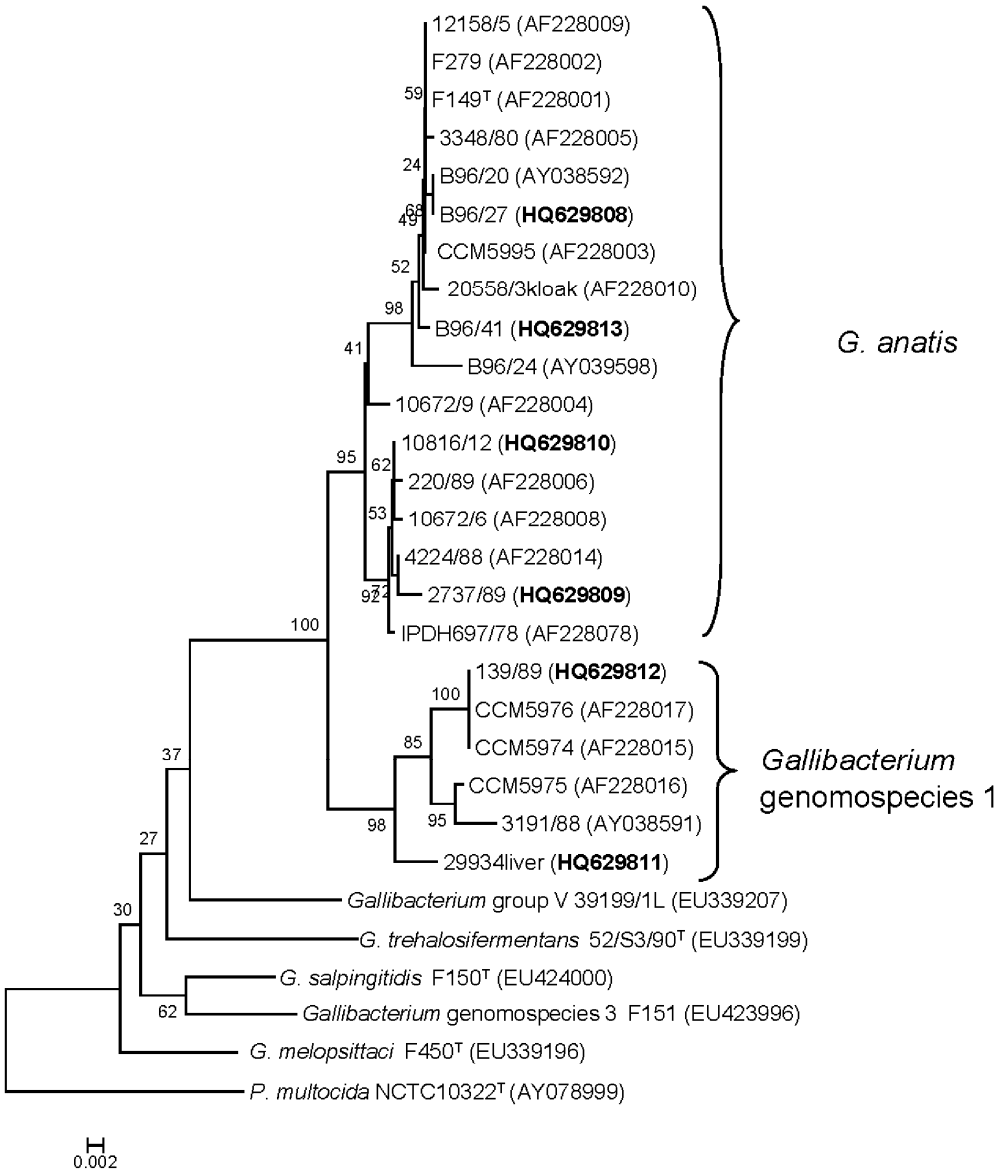

Fig. 3c. recN

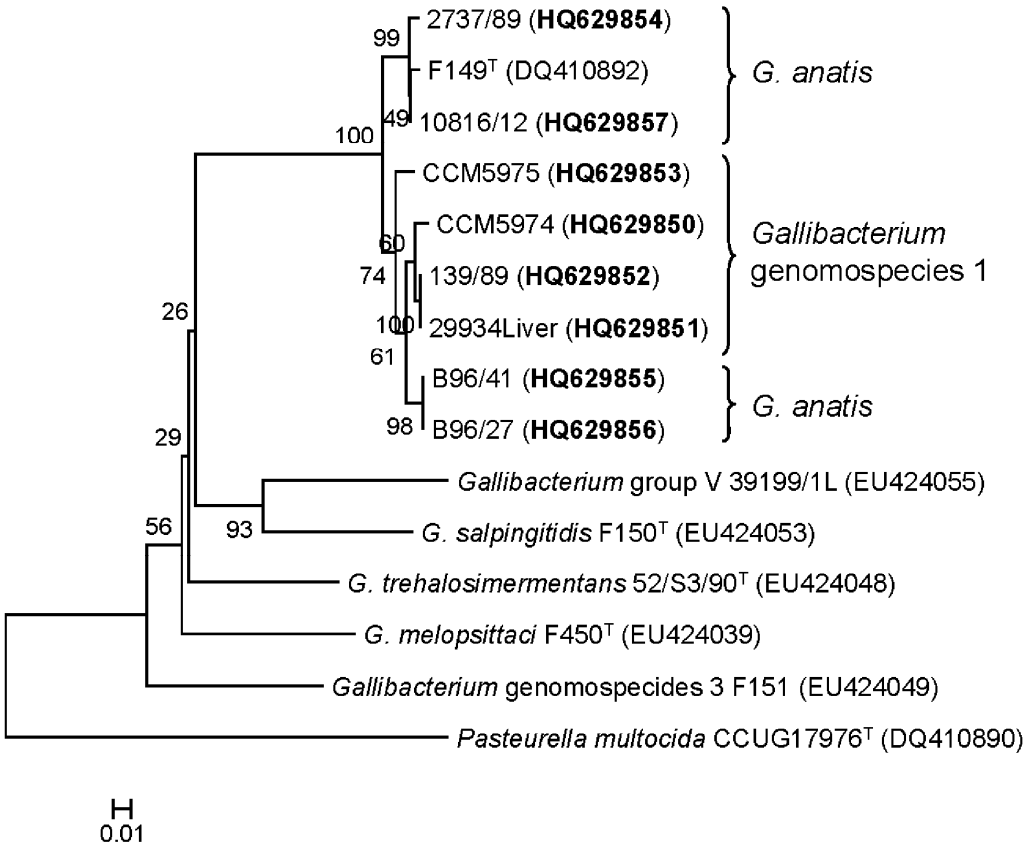
2737/89 (HQ629854)
F149T (DQ410892)
10816/12 (HQ629857)
G. anatis
CCM5975 (HQ629853)
CCM5974 (HQ629850)
139/89 (HQ629852)
29934Liver (HQ629851)
Gallibacterium genomospecies 1
B96/41 (HQ629855)
B96/27 (HQ629856)
G. anatis
99
49
100
60
74
100
61
98
26
29
56
93
Gallibacterium group V 39199/1L (EU424055)
G. salpingitidis F150T (EU424053)
G. trehalosimermentans 52/S3/90T (EU424048)
G. melopsittaci F450T (EU424039)
Gallibacterium genomospecides 3 F151 (EU424049)
Pasteurella multocida CCUG17976T (DQ410890)
H
0.01

Fig. 3d. infB

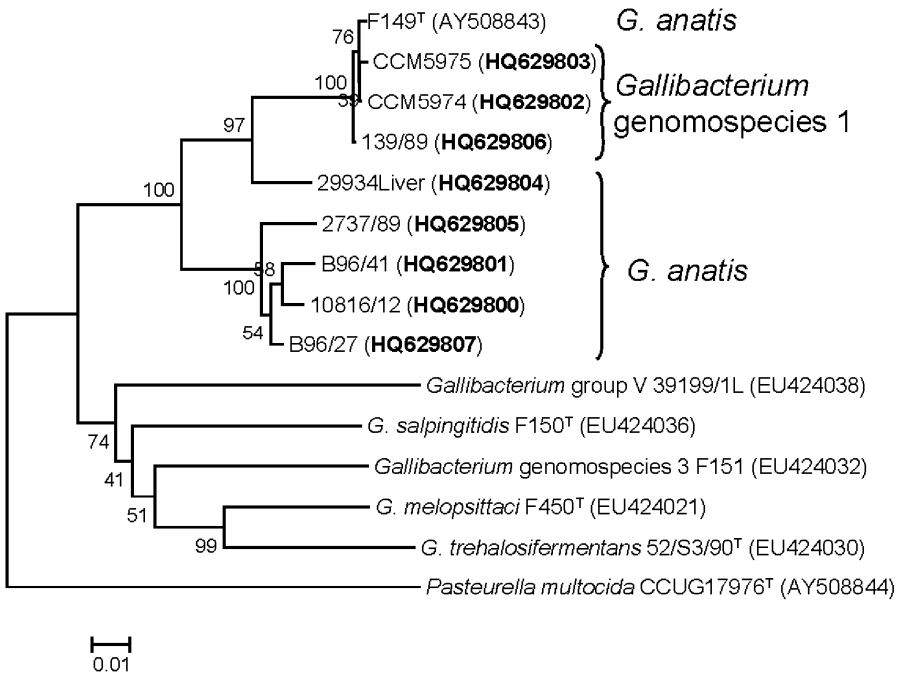

Figure 3.

Phylogenetic relationships between members of *Gallibacterium* as investigated based on neighbor joining analysis of partial *rpoB* gene sequences (a), nearly full length 16S rRNA gene sequences (b), partial *recN* gene sequences (c) and partial *infB* gene sequences (d). Supports for monophyletic groups by bootstrap-analysis are indicated as numbers out of 100. The strain numbers with DBJ/EMBL/GenBank accession numbers marked in bold have been sequenced in the present investigation. The scale bar represents sequence variation considering the model for nucleotide substitution (Jukes & Cantor) and algorithm (Neighbor Joining) used in the analysis.

Discussion

The genus *Gallibacterium* includes a very diverse group of bacteria that vary in pheno- and genotypic characteristics, independent of hosts range, geographical location and time of isolation explaining the difficulties in classification and identification of taxa making up the genus *Gallibacterium.* So far, classification and identification of *Gallibacterium* species have been based upon phenotypic and genotypic methods, including DNA-DNA hybridisation, PFGE, AFLP and 16S rRNA sequencing (Bisgaard, 1977; Bisgaard, 1993; Christensen *et al.*, 2003).

More recently, a number of strains were characterized by *infB, recN* and *rpoB* gene sequencing (Bisgaard *et al.*, 2009). However, many of these methods are complex, costly and often require days to complete. In addition, inconsistent results may be obtained with different genes as demonstrated in the present paper. The *rpoB* gene sequence is strongly conserved within the various species of the family of *Pasteurellaceae* and the resolution is generally greater than that of the 16S rRNA sequence, but still does not allow the separation of very closely related species (Korczak *et al.*, 2004). This study investigated if MALDI-TOF MS allows unambiguous separation and identification of *Gallibacterium* species recognised as mainly avian pathogens. With the exception of the strain named 39199/1L (group V), the overall congruency between MALDI-TOF MS and MLSA was good. This observation is in agreement with results reported by Tanigawa *et al,* (2010) who stated that the MALDI-TOF MS results was nearly identical to genotypic identification (16S rRNA and recA gene sequence and AFLP) for discriminating species and subspecies in the genus *Lactococcus*. According to Christensen *et al.* (2003), AFLP and DNA reassociation data seem to indicate the existence of only one additional genomospecies of *Gallibacterium* (strain CCM5976). MALDI-TOF MS results did not support that CCM5976 belong to an independent species (Figure 2). Neither did sequencing of *rpoB* (Figure 3 a), while 16S rRNA data classified CCM5976 with genomospecies 2. Korczak *et al.* (2004) noted that within the family *Pasteurellaceae*, discrepancies were observed for some species between 16S rRNA and *rpoB* gene-based phylogenies. The discrepancy that was observed between MLSA and MALDI-TOF MS of *Gallibacterium* group V remains unexplained. However, comparison of whole cell protein profiling and DNA reassociation of strains classified as taxon 2 or 3 also demonstrated deviations

(Bisgaard, 1993). Apart from strains 2737/89 and 10816/12, the other strains of cluster 2 (Figure 2) seem to represent a new species of *Gallibacterium* previously outlined as genomospecies 1 by genetic methods (Christensen *et al.*, 2003). Clustering of *G. salpingitidis* with *Gallibacterium* genomospecies 3 (F151) (cluster 3) was also confirmed by 16S rRNA and *rpoB* sequencing results. However, whole-genome similarity between these taxa calculated from *recN* sequences showed that these taxa represent different species (Bisgaard *et al.*, 2009). Finally, three strains isolated from bovine lungs (B96/41, B96/20 and B96/27) exhibited extensive variation between their *rpoB* and their *infB*, 16S rRNA and *recN* gene sequence. The reason behind this observation remains to be investigated.

An increased number of papers comparing the use of MALDI/Biotyper with the standard methods for identification in clinical diagnostic laboratories have been published recently (Carbonnelle *et al.*, 2010; Giebel *et al.*, 2010). Bizzini *et al*. (2010) compared MALDI/Biotyper to the conventional phenotypic methods for identification of 1,371 routine isolates, in which 93.2% were identified to species level with both methods. Poor 16S rRNA sequence similarity between the species *Nocardia paucivorans* and *N. transvalensis* was noted even though MALDI-TOF MS dendrogram showed that their mass signal patterns are closely related (Verroken *et al.*, 2010). On the other hand, MALDI-TOF MS was used to support the data from 16S rRNA and *rpoB* gene sequence data to indicate that *Acinetobacter bereziniae* and *A. guillouiae* represent distinct groups within the genus *Acinetobacter* (Nemec *et al.*, 2010).

Table 1. Reference strains used in this study.

Strain name	Source of isolation	Results			GenBank accession number			
		Biochemical/ physiological	MALDI-TOF MS[a]	Genotypic[b]	16S rRNA	rpoB	recN	infB
2737/89	Turkey, septicaemia	*G. anatis* biovar 11	*G.* genomospecies 1	*G. anatis*	**HQ629809**	**HQ629815**	**HQ629854**	**HQ629805**
220/89	No data	*G. anatis* biovar 24	*G. anatis*	*G. anatis*	AF228006[c]			
34346ov	Chicken, ovary	*G. anatis* biovar 4	*G. anatis*					
F281	Duck	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629825**		
F282	Duck	*G. anatis* biovar anatis	*G. anatis*					
3911/90	Rosella, septicaemia	*G. anatis* biovar 4	*G. anatis*					
12656/12L	Chicken, septicaemia	*G. anatis* biovar 4	*G. anatis*					
10672/9Salp	Chicken, salpingitis	*G. anatis* biovar 4	*G. anatis*	*G. anatis*	AF228004[c]			
5821/88	A. militensis, septicaemia	*G. anatis* biovar 4	*G. anatis*					
10814/13Salp	Chicken, salpingitis	*G. anatis* biovar 4	*G. anatis*					
1779/90	Guinea fowl, septicaemia	*G. anatis* biovar 4	*G. anatis*	*G. anatis*		**HQ629817**		
4258/90 [d]	Partridge, septicaemia	*G. anatis* biovar 4	*G. anatis*					
2125/89	Chicken,	*G. anatis* biovar 11	*G. anatis*	*G. anatis*		**HQ629816**		

	septicaemia									
121/89	Pheasant, septicaemia	*G. anatis* biovar 18	*G. anatis*							
20558/3kloak	Goose	*G. anatis* biovar 19	*G. anatis*	*G. anatis*	AF228010[c]					
1746/87	Chicken, septicaemia	*G. anatis* biovar 18	*G. anatis*							
CCM5995	Chicken	*G. anatis* biovar 20	*G. anatis*	*G. anatis*	AF228003[c]	**HQ629824**				
10816/12	Salpingitis, chicken	*G. anatis* biovar 18	*G.* genomospecies 1	*G. anatis*	**HQ629810**	**HQ629845**	**HQ629857**	**HQ629800**		
444/89	Budgerigar, septicaemia	*G. anatis* biovar 18	*G. anatis*	*G. anatis*		**HQ629849**				
10673/1Salp	Chicken, salpingitis	*G. anatis* biovar 1	*G. anatis*							
3348/80	Goose, septicaemia	*G. anatis* biovar 17	*G. anatis*	*G. anatis*	AF228005[c]	**HQ629828**				
3268/87	Chicken, septicaemia	*G. anatis* biovar 18	*G. anatis*							
4224/88	No data	*G. anatis* biovar 22	*G. anatis*	*G. anatis*	AF228014[c]	**HQ629822**				
1939/89	Budgerigar, septicaemia	*G. anatis* biovar 18	*G. anatis*							
IPDH 697/78	Chicken	*G. anatis* biovar 15	*G. anatis*	*G. anatis*	AF228007[c]	**HQ629827**				
14542/2L	Chicken, septicaemia	*G. anatis* biovar 12	*G. anatis*	*G. anatis*		**HQ629826**				
42447/2L	Chicken, septicaemia	*G. anatis* biovar 12	*G. anatis*							
442/89	Budgerigar,	*G. anatis* biovar 18	*G. anatis*							

	septicaemia							
P6Organs	Bovine	*G. anatis* biovar anatis	*G. anatis*					
99066106	Duck Brain	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629834**		
H3Lung	Bovine	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629847**		
B96/24	Bovine Brain	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*	AY039598	**HQ629844**		
HPA126	Duck	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629842**		
B96/21	Pig Brain	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629832**		
2589/89	Chicken, septicaemia	*G. anatis* biovar 1	*G. anatis*	*G. anatis*		**HQ629839**		
B96/26	Bovine	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629833**		
1307/89	Budgerigar, septicaemia	*G. anatis* biovar 1	*G. anatis*					
66Liver	Goose	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629843**		
F280	Duck	*G. anatis* biovar anatis	*G. anatis*					
P. sp. 38	Chicken, yolk	*G. anatis* biovar 12	*G. anatis*	*G. anatis*		**HQ629821**		
H2Lung	Bovine	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*		**HQ629848**		
6263/88	Chicken, septicaemia	*G. anatis* biovar 12	*G. anatis*	*G. anatis*		**HQ629837**		
HIM996-9	Duck	*G. anatis* biovar anatis	*G. anatis*					
10672/6	Chicken, salpingitis	*G. anatis* biovar 1	*G. anatis*	*G. anatis*	AF228008[c]	**HQ629830**		
12158/5	Chicken, salpingitis	*G. anatis* biovar 3	*G. anatis*	*G. anatis*	AF228009[c]	**HQ629835**		
646/89	Chicken, septicaemia	*G. anatis* biovar 1	*G. anatis*	*G. anatis*		**HQ629823**		
5693/88	Chicken,	*G. anatis* biovar 12	*G. anatis*	*G. anatis*		**HQ629814**		

	septicaemia									
4851/87	Budgerigar, septicaemia	*G. anatis* biovar 12	*G. anatis*							
3076/88	Duck, septicaemia	*G. anatis* biovar 17	*G. anatis*	*G. anatis*	**HQ629840**					
F279	Duck	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*	AF228002[c]					
F149[T](CCUG15563[T])	Duck	*G. anatis* biovar anatis	*G. anatis*	*G. anatis*	AF228001[c]	AY314032[c]	DQ410892[c]	AY508843[c]		
3109/88	Budgerigar, septicaemia	*G. anatis* biovar 12	*G. anatis*	*G. anatis*	**HQ629838**					
CCM5974[d]	Chicken	*G. anatis* biovar 8	*G.* genomospecies 1	*G.* genomosp. 1 by *rpoB* and 16S rRNA. *G. anatis* by *recN* and *infB*	AF228015[c]	AY314033[c]	**HQ629850**	**HQ629802**		
CCM5975[d]	Chicken	*G. anatis* biovar 5	*G.* genomospecies 1	*G.* genomosp. 1 by *rpoB* and 16S rRNA. *G. anatis* by *recN* and *infB*	AF228016[c]	**HQ629831**	**HQ629853**	**HQ629803**		
29934Liver	Chicken, septicaemia	*G. anatis* biovar 24	*G.* genomospecies 1	*G.* genomosp. 1 by *rpoB* and 16S rRNA. *G. anatis* by *recN* and *infB*	**HQ629811**	**HQ629836**	**HQ629851**	**HQ629804**		
139/89	Chicken,	*G. anatis* biovar 23	*G.* genomospecies 1	*G.* genomosp. 1 by	**HQ629812**	**HQ629841**	**HQ629852**	**HQ629806**		

	septicaemia			*rpoB* and 16S rRNA. *G. anatis* by *recN* and *infB*				
3191/88	Chicken, septicaemia	*G. anatis* biovar 8	*G. anatis*	*G.* genomosp. 1	AY038591[c]	**HQ629820**		
F151 [d]	Duck, salpingitis	*Gallibacterium* variant (*G.* genomospecies 3)	Clusters with *G. salpingitidis*	*G. salpingitidis* by *rpoB* and 16S rRNA. *G.* genomosp. 3 by *recN* and *infB*	EU423996[c]	EU424014[c]	EU424049[c]	EU424032[c]
52/S3/90^{T} [d](CCUG 55631^{T})	Budgerigar, septicaemia	*G. trehalosifermentans*	*G. trehalosifermentans*	*G. trehalosifermentans* by 16S rRNA and *recN*	EU339199[c]	EU424012[c]	EU424048[c]	EU424030[c]
F450^{T} [d](CCUG 36331^{T})	Parakeet, septicaemia	*G. melopsittaci*	*G. melopsittaci*	*G. melopsittaci* by 16S rRNA and *recN*	EU339196[c]	EU424003[c]	EU424039[c]	EU424021[c]
F150^{T} [d](CCUG 15564^{T})	Duck, salpingitis	*G. salpingitidis*	*G. salpingitidis* (clusters with genomospecies 3 F151 strain)	*G. salpingitidis*	EU424000[c]	EU424018[c]	EU424053[c]	EU424036[c]
39199/1L	Pheasant, pneumonia	*Gallibacterium* group V	*G. anatis*	*Gallibacterium* group V	EU339207[c]	EU424020[c]	EU424055[c]	EU424038[c]
CCM5976	Chicken	*G. anatis* biovar 9 (genomospecies 2)	*G. anatis*	*G. anatis* by *rpoB* *G.* genomosp. 2 by 16S rRNA	AF228017[c]	**HQ629846**		

B96/41	Bovine Lung	*G. anatis* biovar anatis	*G. anatis*	G. genomosp. 3 by *rpoB* *G. anatis* by 16S rRNA, *recN* and *infB*	**HQ629813**	**HQ629819**	**HQ629855**	**HQ629801**
B96/20 [d]	Bovine Lung	*G. anatis* biovar anatis	*G. anatis*	*G. salpingitidis* *rpoB* *G. anatis* 16S rRNA	AY038592[c]	**HQ629829**		
B96/27	Bovine Lung	*G. anatis* biovar anatis	*G. anatis*	*G. salpingitidis* by *rpoB* *G. anatis* by 16S rRNA, *recN* and *infB*	**HQ629808**	**HQ629818**	**HQ629856**	**HQ629807**

Abbreviations: G.: *Gallibacterium*, G. genomosp.: *Gallibacterium* genomospecies
[a] Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
[b] Results from sequencing one or more genes as indicated with accession number.
[c] Gene sequence published in previous publications (9,16,22).
[d] Strains used for testing the reproducibility of MALDI-TOF MS results under different conditions.

Bacteria rapidly respond to environmental changes that might induce changes in the protein profile. Thus, the reproducibility of MALDI-TOF MS results was investigated under different growth and storage conditions of bacteria, relevant in a clinical laboratory. Reliable identification was possible when bacteria were stored at 4°C degree for up to 8 days. However, if bacteria were grown longer then 72hrs, unreliable identification was seen. This might be explained as a result of depletion of nutrients imposing changes in the protein profile (Valentine *et al.*, 2005). Arnold *et al*. (1999) also stated that the time of incubation should be carefully controlled if MALDI-TOF MS is used for bacterial identification.

In conclusion, this is the first study employing the MALDI-TOF MS fingerprinting technique for the in-depth analysis of *Gallibacterium* species. MALDI-TOF MS clearly discriminated between the four recognised *Gallibacterium* type strains and a possible fifth species *Gallibacterium* genomospecies 1, in agreement with previous findings. However, several minor discrepancies were observed between MALDI-TOF MS and MLSA, the causes of which remains to be investigated. MALDI-TOF MS fingerprinting, however, represents a fast and reliable method for the identification and differentiation of four recognised *Gallibacterium* species and possible fifth species *Gallibacterium* genomospecies 1, with applications in clinical diagnostics.

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References

1. **Alispahic, M., Hummel, K., Jandreski-Cvetkovic, D., Nobauer, K., Razzazi-Fazeli, E., Hess, M. & Hess, C. (2010).** Species-specific identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis. *J Med Microbiol* **59,** 295-301.

2. **Angen, O., Ahrens, P., Kuhnert, P., Christensen, H. & Mutters, R. (2003).** Proposal of *Histophilus somni* gen. nov., sp nov for the three species incertae sedis '*Haemophilus somnus*', '*Haemophilus agni*' and '*Histophilus ovis*'. *Int J Syst Evol Microbiol* **53,** 1449-1456.

3. **Arnold, R. J., Karty, J. A., Ellington, A. D. & Reilly, J. P. (1999).** Monitoring the growth of a bacteria culture by MALDI-MS of whole cells. *Anal Chem* **71,** 1990-1996.

4. **Bisgaard, M. (1977).** Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. characterisation of 213 strains. *Avian Pathol* **6,** 285-292.

5. **Bisgaard, M. (1993).** Ecology and significance of *Pasteurellaceae* in animals. *Zbl Bakt-Int J Med M* **279,** 7-26.

6. **Bisgaard, M. & Dam, A. (1981).** Salpingitis in poultry. II. Prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. *Nordisk Vet Med* **33,** 81-89.

7. **Bisgaard, M., Korczak, B. M., Busse, H. J., Kuhnert, P., Bojesen, A. M. & Christensen, H. (2009).** Classification of the taxon 2 and taxon 3 complex of Bisgaard within *Gallibacterium* and description of *Gallibacterium melopsittaci* sp nov., *Gallibacterium trehalosifermentans* sp nov and *Gallibacterium salpingitidis* sp nov. *Int J Syst Evol Microbiol* **59,** 735-744.

8. **Bizzini, A., Durussel, C., Bille, J., Greub, G. & Prod'hom, G. (2010).** Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. *J Clin Microbiol* **48,** 1549-1554.

9. **Bojesen, A. M., Christensen, H., Nielsen, O. L., Olsen, J. E. & Bisgaard, M. (2003a).** Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA in situ hybridization. *J Clin Microbiol* **41,** 5167-5172.

10. **Bojesen, A. M., Torpdahl, M., Christensen, H., Olsen, J. E. & Bisgaard, M. (2003b).** Genetic diversity of *Gallibacterium anatis* isolates from different chicken flocks. *J Clin Microbiol* **41,** 2737-2740.

11. **Bojesen, A. M., Vazquez, M. E., Robles, F., Gonzalez, C., Soriano, E. V., Olsen, J. E. & Christensen, H. (2007).** Specific identification of *Gallibacterium* by a PCR using primers targeting the 16S rRNA and 23S rRNA genes. *Vet Microbiol* **123,** 262-268.

12. **Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin, B., Beretti, J. L., Ferroni, A., Gutmann, L. & Nassif, X. (2010).** MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clin Biochem* doi:10.1016/j.clinbiochem.2010.06.017.

13. **Christensen, H., Bisgaard, M., Angen, O. & Olsen, J. E. (2002).** Final classification of Bisgaard taxon 9 as *Actinobacillus arthritidis* sp. nov. and recognition of a novel genomospecies for equine strains of *Actinobacillus lignieresii*. *Int J Syst Evol Microbiol* **52,** 1239-1246.

14. **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 genomospecles within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53,** 275-287.

15. **Christensen, H., Kuhnert, P., Olsen, J. E. & Bisgaard, M. (2004).** Comparative phylogenies of the housekeeping genes atpD, infB and rpoB and the 16S rRNA gene within the *Pasteurellaceae*. *Int J Syst Evol Microbiol* **54,** 1601-1609.

16. **Claydon, M. A., Davey, S. N., EdwardsJones, V. & Gordon, D. B. (1996).** The rapid identification of intact microorganisms using mass spectrometry. *Nat Biothechnol* **14,** 1584-1586.

17. **Euzeby, J. P. (1997).** List of bacterial names with standing in nomenclature: A folder available on the Internet. *Int J Syst Bacteriol* **47,** 590-592.

18. **Gerlach, H. (1977).** Significance of *Pasteurella haemolytica* in poultry farms. *Prakt Tierarzt* **58,** 324-328.

19. **Giebel, R., Worden, C., Rust, S. M., Kleinheinz, G. T., Robbins, M. & Sandrin, T. R. (2010).** Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): applications and challenges. *Adv Appl Microbiol* **71,** 149-184.

20. **Jordan, F. T. W., Williams, N. J., Wattret, A. & Jones, T. (2005).** Observations on salpingitis, peritonitis and salpingoperitonitis in a layer breeder flock. *Vet Record* **157,** 573-577.

21. **Korczak, B., Christensen, H., Emler, S., Frey, J. & Kuhnert, P. (2004).** Phylogeny of the family *Pasteurellaceae* based on rpoB sequences. *Int J Syst Evol Microbiol* **54,** 1393-1399.

22. **Kristensen, B. M., Frees, D. & Bojesen, A. M. (2010).** GtxA from *Gallibacterium anatis*, a cytolytic RTX-toxin with a novel domain organisation. *Vet Res* **41,** 25.

23. **Kuhnert, P., Frey, J., Lang, N. P. & Mayfield, L. (2002).** Phylogenetic analysis of *Prevotella nigrescens*, *Prevotella intermedia* and *Porphyromonas gingivalis* clinical strains reveals a clear species clustering. *Int J Syst Evol Microbiol* **52,** 1391-1395.

24. **Kuhnert, P., Korczak, B., Falsen, E., Straub, R., Hoops, A., Boerlin, P., Frey, J. & Mutters, R. (2004).** *Nicoletella semolina* gen. nov., sp. nov., a new member of *Pasteurellaceae* isolated from horses with airway disease. *J Clin Microbiol* **42,** 5542-5548.

25. **Kuhnert, P. & Korczak, B. M. (2006).** Prediction of whole-genome DNA-DNA similarity, determination of G+C content and phylogenetic analysis within the family *Pasteurellaceae* by multilocus sequence analysis (MLSA). *Microbiol -Sgm* **152,** 2537-2548.

26. **Mollet, C., Drancourt, M. & Raoult, D. (1997).** rpoB sequence analysis as a novel basis for bacterial identification. *Mol Microbiol* **26,** 1005-1011.

27. **Mushin, R., Weisman, Y. & Singer, N. (1980).** *Pasteurella haemolytica* found in the respiratory-tract of fowl. *Avian Dis* **24,** 162-168.

28. **Nemec, A., Musilek, M., Sedo, O., De Baere, T., Maixnerova, M., van der Reijden, T. J. K., Zdrahal, Z., Vaneechoutte, M. & Dijkshoorn, L. (2010).** *Acinetobacter bereziniae* sp nov and *Acinetobacter guillouiae* sp nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. *Int J Syst Evol Microbiol* **60,** 896-903.

29. **Neubauer, C., De Souza-Pilz, M., Bojesen, A. M., Bisgaard, M. & Hess, M. (2009).** Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders. *Avian Pathol* **38,** 1-7.

30. **Rice, P., Longden, I. & Bleasby, A. (2000).** EMBOSS: The European molecular biology open software suite. *Trends Genet* **16,** 276-277.

31. **Sauer, S., Freiwald, A., Maier, T., Kube, M., Reinhardt, R., Kostrzewa, M. & Geider, K. (2008).** Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS One* **3,** e2843.

32. **Suh, M. J. & Limbach, P. A. (2004).** Investigation of methods suitable for the matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins from ribonucleoprotein complexes. *Eur J Mass Spectrom (Chichester, Eng)* **10,** 89-99.

33. **Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24,** 1596-1599.

34. **Tanigawa, K., Kawabata, H. & Watanabe, K. (2010).** Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol* **76,** 4055-4062.

35. **Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25,** 4876-4882.

36. **Valentine, N., Wunschel, S., Wunschel, D., Petersen, C. & Wahl, K. (2005).** Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. *Appl Environ Microbiol* **71,** 58-64.

37. **Verroken, A., Janssens, M., Berhin, C., Bogaerts, P., Huang, T. D., Wauters, G. & Glupczynski, Y. (2010).** Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of *Nocardia* species. *J Clin Microbiol* **48,** 4015-4021.

3.3. MALDI-TOF MS reveals different clonal lineages of *Gallibacterium anatis* within a defined population

Manuscript in preparation

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Abstract

Gallibacterium anatis has been suggested to have a causal role in the salpingitis/peritonitis complex in chickens, beside its isolation from the respiratory tract. MALDI-TOF MS correctly identified 184 *G. anatis* isolates from different organs of layers kept in alternative husbandry systems and showing reproductive disorders. In addition, MALDI/Biotyper based clustering revealed that *G. anatis* isolates obtained from upper respiratory tract, reproductive tract, heart, liver and spleen of the same bird belong to the same clonal complex. However, different clonal lineages of *G. anatis* were observed between flocks, indicating each flock was infected with a single clone, supporting previous findings by genotypic methods.

Introduction

The genus *Gallibacterium* belongs to the family *Pasteurellaceae* Pohl 1981 and consists of four recognized species, *Gallibacterium anatis* the type species of the genus *Gallibacterium*, *Gallibacterium salpingitis*, *Gallibacterium melopsittaci*, and *Gallibacterium trehalosifermentans* [1,2]. Depending on the level of biosecurity, *G. anatis* might constitute part of the normal upper respiratory - and the lower genital tract microflora of chickens [3,4]. In addition, these organisms have been isolated from a range of pathological lesions in poultry, including septicaemia, oophoritis, salpingitis, peritonitis and respiratory tract lesions [5-9].

Whole-cell matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) represents an emerging technology for identification and typing of bacteria [10-13]. The present study uses MALDI-TOF MS to identify 184 clinical strains of *G. anatis* that were isolated from respiratory tract, heart, liver, spleen, intestine and reproductive tract from laying birds with reproductive disorders (three to eight birds per flock), kept in alternative husbandry systems. Furthermore, the ability of MALDI/Biotyper software to recognize clonally related *G. anatis* strains (i.e. sub-typing) isolated from different organs as well as from different flocks was investigated. Thus, to enable identification of possible specific *G. anatis* clones.

Materials and Methods

All clinical isolates of *G. anatis* were grown on Columbia agar (COS) containing 5% sheep blood (BioMerieux, Vienna, Austria), at 37°C for 24 hours under microaerobic conditions. All isolates demonstrated a wide β–haemolytic zone (1 to 2 mm). A field isolate of *Pasteurella multocida* (08/14290) was identified by phenotypic methods and confirmed by MALDI/Biotyper reference database version 3, and used as an out-group in the dendrogram. Sample preparation for MALDI-TOF MS, as well as, parameters and data visualization and analysis were performed as previously described in detail [14]. Bacterial acid soluble proteins were extracted using formic acid (70%) and acetonitrile according to the standard protocol from Bruker. One µl of each bacterial extract was spotted five times onto the MALDI target plate and air dried. Afterwards, 2 µl of matrix solution (alpha-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluor acetic acid) were overlaid on each sample and dried again. All steps were performed at room temperature.

Results and Discussion

The Bruker reference database version 3 consists of microorganisms usually relevant in human clinical microbiology laboratories, though only one *Gallibacterium* species (*G. anatis* DSM 16844/DSM) was present. Hence, veterinary important bacteria such as *Gallibacterium* have not been generally evaluated using MALDI-TOF MS, therefore, restricting the practical reach of this new technology. For this reason, firstly the construction of a database in the MALDI/Biotyper with the four *Gallibacterium* type strains (*G. anatis* (F149^T), *G. melopsittaci* (F450^T), *G. trehalosifermentans* (52/33/90^T) *and G. salpingitidis* (F150^T)) and *Gallibacterium* genomospecies 1 (CCM5974). These strains were in-depth characterised by biochemical/physiological properties, genotypic methods and MALDI-TOF MS [15].

For identification of clinical isolates by MALDI-TOF MS, a generated peak list was matched against the established reference library using the integrated pattern-matching algorithm of MALDI/Biotyper software version 2.0. Briefly, the software calculates log(score) values, that is, a log(score) between 1.7 and 2.0 represents genus

identification. Log(score) value of ≥ 2.0 represents an identification at species level. Anything less then 1.7 log(score) was rated as not identifiable by the software.

Table 1. Identification results for the 184 clinical isolates using MALDI-TOF MS/Biotyper and *Gallibacterium* specific PCR

			PCR [a]	MALDI-TOF MS/Biotyper	
No.	**Designation***	**Organ**	**Species ID**	**Detected Species**	**log(Score)[b]**
1	06/0956-1	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.315
2	06/0956-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.312
3	06/0956-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.418
4	06/0956-4	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.463
5	06/0956-4	Trachea	*Gallibacterium anatis*	*G. anatis*	2.283
6	06/0956-5	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.421
7	06/0956-5	Trachea	*Gallibacterium anatis*	*G. anatis*	2.517
8	06/0956-6	Trachea	*Gallibacterium anatis*	*G. anatis*	2.302
9	06/0956-6	Lung	*Gallibacterium anatis*	*G. anatis*	2.411
10	06/0956-7	Trachea	*Gallibacterium anatis*	*G. anatis*	2.537
11	06/0956-8	Trachea	*Gallibacterium anatis*	*G. anatis*	2.455
12	06/0956-8	Liver k.	*Gallibacterium anatis*	*G. anatis*	2.565
13	06/0956-8	Liver g	*Gallibacterium anatis*	*G. anatis*	2.496
14	06/0957-1	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.503
15	06/0957-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.121
16	06/0958-1	Ovary k.	*Gallibacterium anatis*	*G. anatis*	2.542
17	06/0958-1	Ovary g.	*Gallibacterium anatis*	*G. anatis*	2.555
18	06/0958-1	Trachea k.	*Gallibacterium anatis*	*G. anatis*	2.517
19	06/0958-1	Trachea g.	*Gallibacterium anatis*	*G. anatis*	2.479
20	06/0958-1	Oviduct k.	*Gallibacterium anatis*	*G. anatis*	2.564
21	06/0958-1	Oviduct g.	*Gallibacterium anatis*	*G. anatis*	2.441
22	06/0958-1	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.494
23	06/0958-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.524
24	06/0958-2	Lung	*Gallibacterium anatis*	*G. anatis*	2.597
25	06/0958-2	Oviduct	*Gallibacterium anatis*	*G. anatis*	2.295
26	06/0958-2	Ovary	*Gallibacterium anatis*	*G. anatis*	2.455
27	06/0958-3	Ovary	*Gallibacterium anatis*	*G. anatis*	2.308
28	06/0958-4	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.390
29	06/0958-4	Lung	*Gallibacterium anatis*	*G. anatis*	2.527
30	06/0958-4	Oviduct	*Gallibacterium anatis*	*G. anatis*	2.330

31	06/0958-4	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.538
32	06/1321-1	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.327
33	06/1321-1	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.273
34	06/1321-2	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.376
35	06/1321-2	Heart [c]	*Gallibacterium anatis*	*G. anatis*	2.411
36	06/1321-2	Liver [c]	*Gallibacterium anatis*	*G. anatis*	2.444
37	06/1321-2	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.410
38	06/1321-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.583
39	06/1321-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.390
40	06/1321-3	Choana	*Gallibacterium anatis*	*G. anatis*	2.410
41	06/1321-3	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.448
42	06/1321-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.633
43	06/1321-3	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.459
44	06/1321-3	Spleen	*Gallibacterium anatis*	*G. anatis*	2.570
45	06/1322-1	Oviduct	*Gallibacterium anatis*	*G. anatis*	2.225
46	06/1322-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.439
47	06/1322-3	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.292
48	06/1322-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.412
49	06/1322-3	Oviduct [c]	*Gallibacterium anatis*	*G. anatis*	2.397
50	06/2159-1	Lung	*Gallibacterium anatis*	*G. anatis*	2.272
51	06/2159-1	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.337
52	06/2160-3	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.414
53	06/2161-1	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.364
54	06/2162-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.364
55	06/2162-1	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.341
56	06/2162-1	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.630
57	06/2162-1	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.320
58	06/2162-3	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.397
59	06/2162-3	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.557
60	06/2163-1	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.564
61	06/2163-1	Oviduct [c]	*Gallibacterium anatis*	*G. anatis*	2.524
62	06/2163-2	Choana	*Gallibacterium anatis*	*G. anatis*	2.113
63	06/2163-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.488
64	06/2600-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.531
65	06/2600-1	Ovary	*Gallibacterium anatis*	*G. anatis*	2.487
66	06/2600-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.419
67	06/2600-2	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.315
68	06/2600-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.314
69	06/2600-5	Choana	*Gallibacterium anatis*	*G. anatis*	2.259
70	06/2600-5	Trachea	*Gallibacterium anatis*	*G. anatis*	2.221

71	06/2601-4	Choana	*Gallibacterium anatis*	*G. anatis*	2.614
72	06/2601-4	Trachea	*Gallibacterium anatis*	*G. anatis*	2.370
73	06/2601-5	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.247
74	06/2601-5	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.325
75	06/3358-1	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.455
76	06/3358-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.452
77	06/3358-4	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.239
78	06/3358-4	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.280
79	06/3358-5	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.539
80	06/3358-5	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.379
81	06/3358-5	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.388
82	06/3358-5	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.540
83	06/3358-5	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.360
84	06/3359-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.546
85	06/3359-2	Choana	*Gallibacterium anatis*	*G. anatis*	2.463
86	06/3359-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.431
87	06/3359-3	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.200
88	06/3359-3	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.524
89	06/3359-3	Oviduct [c]	*Gallibacterium anatis*	*G. anatis*	2.397
90	06/3359-3	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.378
91	06/3359-4	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.304
92	06/3359-4	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.570
93	06/3360-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.426
94	06/3360-1	Trachea	*Gallibacterium anatis*	*G. anatis*	2.429
95	06/4296-2	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.388
96	06/4296-2	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.323
97	06/4296-2	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.596
98	06/4296-2	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.467
99	06/4296-4	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.270
100	06/4296-4	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.385
101	06/4296-4	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.397
102	06/4296-4	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.481
103	06/4567-3	Lung	*Gallibacterium anatis*	*G. anatis*	2.287
104	06/4567-3	Choana	*Gallibacterium anatis*	*G. anatis*	2.300
105	06/4567-4	Oviduct	*Gallibacterium anatis*	*G. anatis*	2.324
106	06/4567-5	Choana	*Gallibacterium anatis*	*G. anatis*	2.495
107	06/7415-1	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.587
108	06/7415-3	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.373
109	06/7415-3	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.355
110	06/7415-3	Oviduct [c]	*Gallibacterium anatis*	*G. anatis*	2.438

111	06/7415-4	Heart [c]	*Gallibacterium anatis*	*G. anatis*	2.409
112	06/7415-5	Trachea	*Gallibacterium anatis*	*G. anatis*	2.511
113	06/7415-5	Liver [c]	*Gallibacterium anatis*	*G. anatis*	2.321
114	06/7415-5	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.310
115	06/7416-1	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.385
116	06/7416-1	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.313
117	06/7416-4	Lung g.	*Gallibacterium anatis*	*G. anatis*	2.602
118	06/7416-4	Lung k.	*Gallibacterium anatis*	*G. anatis*	2.413
119	06/7416-4	Spleen g. [c]	*Gallibacterium anatis*	*G. anatis*	2.187
120	06/7416-4	Spleen k.	*Gallibacterium anatis*	*G. anatis*	2.438
121	06/7416-5	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.324
122	06/7416-5	Liver [c]	*Gallibacterium anatis*	*G. anatis*	2.489
123	06/7417-2	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.423
124	06/7417-2	Oviduct [c]	*Gallibacterium anatis*	*G. anatis*	2.475
125	06/7417-4	Heart [c]	*Gallibacterium anatis*	*G. anatis*	2.444
126	06/7417-4	Liver [c]	*Gallibacterium anatis*	*G. anatis*	2.512
127	06/7417-4	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.444
128	06/7417-4	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.496
129	06/7484-1	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.474
130	06/7484-1	Cloaca [c]	*Gallibacterium anatis*	*G. anatis*	2.519
131	06/7484-1	Trachea [c]	*Gallibacterium anatis*	*G. anatis*	2.578
132	06/7484-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.583
133	06/7484-4	Choana [c]	*Gallibacterium anatis*	*G. anatis*	2.428
134	06/7484-4	Duodenum [c]	*Gallibacterium anatis*	*G. anatis*	2.471
135	06/7484-4	Lung [c]	*Gallibacterium anatis*	*G. anatis*	2.384
136	06/7484-4	Spleen [c]	*Gallibacterium anatis*	*G. anatis*	2.369
137	06/7484-4	Ovary [c]	*Gallibacterium anatis*	*G. anatis*	2.382
138	06/9163-5	Trachea	*Gallibacterium anatis*	*G. anatis*	2.561
139	06/9163-5	Choana	*Gallibacterium anatis*	*G. anatis*	2.550
140	06/9163-5	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.425
141	06/9610-1	Trachea	*Gallibacterium anatis*	*G. anatis*	2.571
142	06/9610-2	Choana	*Gallibacterium anatis*	*G. anatis*	2.593
143	06/9610-3	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.559
144	06/9610-4	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.563
145	07/0052-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.556
146	07/0052-1	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.522
147	07/0052-2	Heart	*Gallibacterium anatis*	*G. anatis*	2.174
148	07/0052-2	Ovary	*Gallibacterium anatis*	*G. anatis*	2.247
149	07/0052-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.498
150	07/0052-3	Choana	*Gallibacterium anatis*	*G. anatis*	2.317

151	07/0052-4	Choana	*Gallibacterium anatis*	*G. anatis*	2.478
152	07/0052-5	Spleen	*Gallibacterium anatis*	*G. anatis*	2.526
153	07/0053-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.352
154	07/0053-2	Lung	*Gallibacterium anatis*	*G. anatis*	2.356
155	07/0053-2	Trachea	*Gallibacterium anatis*	*G. anatis*	2.291
156	07/0053-3	Heart	*Gallibacterium anatis*	*G. anatis*	2.320
157	07/0053-3	Ovary	*Gallibacterium anatis*	*G. anatis*	2.294
158	07/0053-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.416
159	07/0053-3	Lung	*Gallibacterium anatis*	*G. anatis*	2.327
160	07/0053-4	Choana	*Gallibacterium anatis*	*G. anatis*	2.301
161	07/0053-5	Choana	*Gallibacterium anatis*	*G. anatis*	2.538
162	07/0053-5	Heart	*Gallibacterium anatis*	*G. anatis*	2.455
163	07/0053-5	Liver	*Gallibacterium anatis*	*G. anatis*	2.423
164	07/0702-1	Choana	*Gallibacterium anatis*	*G. anatis*	2.320
165	07/0702-1	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.433
166	07/0702-1	Heart	*Gallibacterium anatis*	*G. anatis*	2.415
167	07/0702-1	Liver	*Gallibacterium anatis*	*G. anatis*	2.363
168	07/0702-1	Spleen	*Gallibacterium anatis*	*G. anatis*	2.483
169	07/0702-2	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.389
170	07/0702-2	Liver	*Gallibacterium anatis*	*G. anatis*	2.537
171	07/0702-2	Spleen	*Gallibacterium anatis*	*G. anatis*	2.533
172	07/0702-3	Choana	*Gallibacterium anatis*	*G. anatis*	2.436
173	07/0702-3	Cloaca	*Gallibacterium anatis*	*G. anatis*	2.500
174	07/0702-3	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.457
175	07/0702-3	Heart	*Gallibacterium anatis*	*G. anatis*	2.422
176	07/0702-3	Ovary	*Gallibacterium anatis*	*G. anatis*	2.366
177	07/0702-3	Spleen	*Gallibacterium anatis*	*G. anatis*	2.473
178	07/0702-3	Trachea	*Gallibacterium anatis*	*G. anatis*	2.101
179	07/0702-4	Choana	*Gallibacterium anatis*	*G. anatis*	2.438
180	07/0702-4	Liver	*Gallibacterium anatis*	*G. anatis*	2.326
181	07/0702-4	Trachea	*Gallibacterium anatis*	*G. anatis*	2.390
182	07/0702-4	Heart	*Gallibacterium anatis*	*G. anatis*	2.212
183	07/0702-5	Choana	*Gallibacterium anatis*	*G. anatis*	2.531
184	07/0702-5	Duodenum	*Gallibacterium anatis*	*G. anatis*	2.385

Abbreviations: G: *Gallibacterium*, g: big colonies, k: small colonies.

[a] Polymerase chain reaction (PCR) analyses according to the protocol of Bojesen *et al*. [16].

[b] MALDI Biotyper 2 results stand for: species level identification with log(score) values ≥ 2.0 and genus identification with log(score) values between 1.7 and 2.0.

[c] Strains analysed by AFLP [6].

*****Designation = year/flock number-bird number (e.g.06/1321-2)

Based on cross-wise minimum spanning tree (MSP) matching, a dendrogram was created with similar MSPs, resulting in a high matching score value. Each MSP is matched against all MSPs of the analysed set. The list of score values is used to calculate normalised distance values between the analysed species, resulting in a matrix of matching scores. The visualization of the respective relationship between the MSPs is displayed in a dendrogram using the MALDI Biotyper 2.0 software. Species with distance levels under 500 have been described as reliably classified to species level [17].

A defined population of *G. anatis* field isolates were obtained from a two-year study based on strict selection of layer flocks, a standardised protocol for necropsy and for bacteriological investigation from 10 organs of each bird [6]. The present study uses MALDI Biotyper software for identification of the *G. anatis* field isolates and for investigation of their clonal relationships. Ten isolates representing the diversity outlined by MALDI-TOF MS were subjected to sequencing of *rpoB* gene as described recently [15] to verify that all isolates represented *G. anatis.*

Confirmed to previous results from *Gallibacterium*-specific PCR and AFLP [6] MALDI-TOF MS identified all 184 strains correctly (Table 1). DNA sequence comparison of the partial *rpoB* gene sequence of ten strains (06/7417-4 Trachea, 06/1321-2 Liver, 06/0958-2 Oviduct, 06/2162-1 Trachea, 06/3358-5 Choana, 06/7417-4 Liver, 06/0956-4 Duodenum, 06/7484-4 Duodenum, 06/7484-4 Spleen, 07/0053-3 Heart) showed from 0.4 to 1.3 % variation with 0.4 to 1.8 % variation to the type strain of *G. anatis*.

Isolates from some organs, e.g. 06/0956-8 Liver, 06/0956-8 Trachea, 06/0956-8 Oviduct, 06/7416-4 Spleen, demonstrated differences in colony size i.e. some colonies were 1-2 mm while others colonies were only 0.5 mm in diameter. Differences were not

observed in spectra obtained with small and large colonies on MALDI-TOF MS, and both colony sizes were identified as *G. anatis,* even though the small colony size is not typical for *Gallibacterium* [2].

A score oriented MSP dendrogram was generated with 50 *Gallibacterium* isolates originating from 10 flocks and different organs from 14 different birds. In addition, the type strain of *G. anatis* type strain (F149^{T}) and *Pasteurella multocida* 08/14290 were also included in the dendrogram (Fig. 1). The maximum distance between strains of *G. anatis* included in the dendrogram was 300 well below the cut-off value of 500 usually used for species separation. We propose a cut-off value of 100-distance level in the MALDI/Biotyper dendrogram for clone definition. The isolates that group together below 100-distance level are generally isolated from the same flock. In principle, the MALDI-TOF MS method revealed no different clonal lineages of *G. anatis* strains within the same bird, comparing isolates from upper respiratory tract and lesions in reproductive tract, heart, liver and spleen. Thus, supporting earlier data that these *Gallibacterium* isolates are more likely part of the normal flora and potential opportunist pathogen of chickens [18].

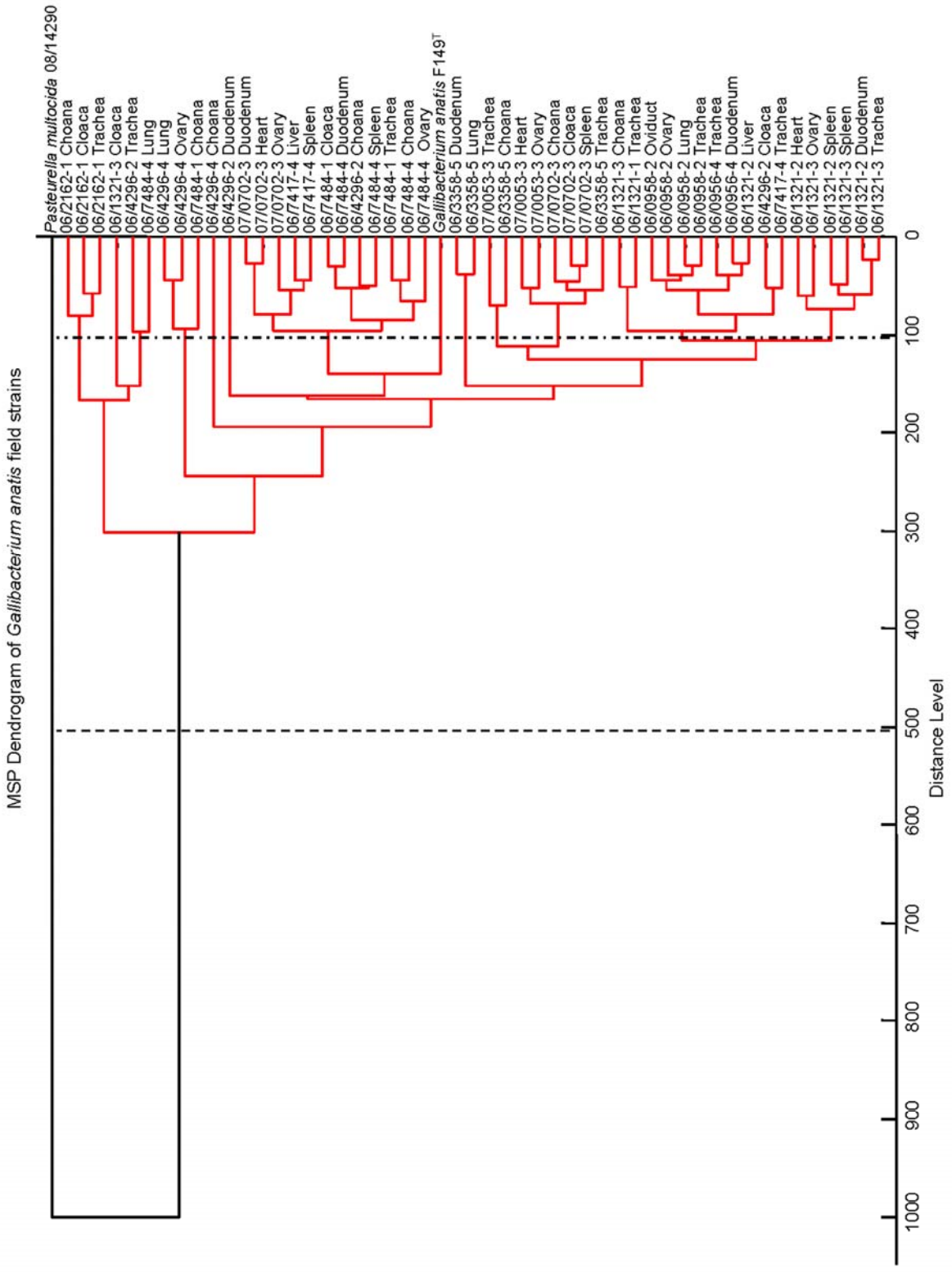

Figure 1.

Classification of *Gallibacterium* clinical strains investigated. Score–oriented (MSP) dendrogram of MALDI-TOF mass spectral profiles generated by the MALDI Biotyper 2. The dendrogram was generated with following settings:

distance measure was set at correlation, linkage at average and score threshold value for a single organism at 600. *Pasteurella multocida* 08/14290 strain was used as an out-group. Strains clustering with distance levels lower than 500 could be classified up to species level [17]. The vertical broken line at 100-distance level indicates the cut-off value for MALDI/Biotyper clone definition.

So far only a few publications have investigated if MALDI-TOF MS and the Biotyper software are capable of revealing different clonal lineages. Dubois *et al.* [19] showed that *Staphylococcus epidermidis* of human or environmental origin clustered separately in the MALDI-TOF MS dendrogram, revealing different clonal lineages according to the origin of the isolates. In addition, Wang *et al.* [20] showed that the MALDI-TOF MS method was capable of determining the geographical origin of commercial honey. The five major hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complexes have also been analysed by MALDI-TOF MS, and it was shown that MALDI-TOF MS based typing allows accurate and reproducible discrimination of major MRSA clonal complexes [13]. However, none of these studies included different isolates from the same individual or populations of animals kept in close contact.

In this study, MALDI/Biotyper based clustering revealed different clonal lineages of *G. anatis* that were isolated from different flocks. This finding confirms previously published genotypic data stating that a single clone might infect a flock and can be isolated from various sites in a bird [6]. Furthermore, AFLP showed that the same clone could be isolated from the mucosal lining of upper respiratory tract and from lesions in the reproductive tract, heart, liver and, spleen, reflecting a continues cascade of the infection [6]. Bojesen *et al.* [18] demonstrate the existence of clonal populations of *Gallibacterium* at the flock level and specific clonal lineages within the same animal.

In conclusion, MALDI-TOF MS and the Biotyper software allowed correct identification of *G. anatis* isolates from birds with reproductive disorders kept in alternative husbandry systems. MALDI/Biotyper also demonstrated different clonal lineages of *G. anatis* that were isolated from different flocks.

Reference List

1. Bisgaard M, Korczak BM, Busse HJ, et al. Classification of the taxon 2 and taxon 3 complex of Bisgaard within *Gallibacterium* and description of *Gallibacterium melopsittaci* sp nov., *Gallibacterium trehalosifermentans* sp nov and *Gallibacterium salpingitidis* sp nov. Int J Syst Evol Microbiol 2009; 59: 735-744.

2. Christensen H, Bisgaard M, Bojesen AM, Mutters R, Olsen JE. 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 genomospecles within *Gallibacterium* gen. nov. Int J Syst Evol Microbiol 2003; 53: 275-287.

3. Bisgaard M. Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. characterisation of 213 strains. Avian Pathol 1977; 6: 285-292.

4. Bojesen AM, Nielsen SS, Bisgaard M. Prevalence and transmission of haemolytic *Gallibacterium* species in chicken production systems with different biosecurity levels. Avian Pathol 2003; 32: 503-510.

5. Jordan FTW, Williams NJ, Wattret A, Jones T. Observations on salpingitis, peritonitis and salpingoperitonitis in a layer breeder flock. Vet Record 2005; 157: 573-577.

6. Neubauer C, De Souza-Pilz M, Bojesen AM, Bisgaard M, Hess M. Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders. Avian Pathol 2009; 38: 1-7.

7. Gerlach H. Significance of *Pasteurella haemolytica* in poultry farms. Prakt Tierarzt 1977; 58: 324-328.

8. Mushin R, Weisman Y, Singer N. *Pasteurella haemolytica* found in the respiratory-tract of fowl. Avian Dis 1980; 24: 162-168.

9. Bisgaard M, Dam A. Salpingitis in poultry. II. Prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. Nordisk Vet Med 1981; 33: 81-89.

10. Dieckmann R, Helmuth R, Erhard M, Malorny B. Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 2008; 74: 7767-7778.

11. Rubbenstroth D, Hotzel H, Knobloch J, et al. Isolation and characterization of atypical *Riemerella columbina* strains from pigeons and their differentiation from *Riemerella anatipestifer*. Vet Microbiol 2010; doi:10.1016/j.vetmic.2010.06.008.

12. Giebel RA, Fredenberg W, Sandrin TR. Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Water Res 2008; 42: 931-940.

13. Wolters M, Rohde H, Maier T, et al. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 2010; doi:10.1016/j.ijmm.2010.06.002.

14. Alispahic M, Hummel K, Jandreski-Cvetkovic D, et al. Species-specific identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis. J Med Microbiol 2010; 59: 295-301.

15. Alispahic M, Christensen Henrik, Hess Claudia, et al. Identification of *Gallibacterium* species using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry confirmed multilocus sequence analysis. submitted for publication 2011.

16. Bojesen AM, Vazquez ME, Robles F, et al. Specific identification of *Gallibacterium* by a PCR using primers targeting the 16S rRNA and 23S rRNA genes. Vet Microbiol 2007; 123: 262-268.

17. Sauer S, Freiwald A, Maier T, et al. Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS One 2008; 3: e2843.

18. Bojesen AM, Torpdahl M, Christensen H, Olsen JE, Bisgaard M. Genetic diversity of *Gallibacterium anatis* isolates from different chicken flocks. J Clin Microbiol 2003; 41: 2737-2740.

19. Dubois D, Leyssene D, Chacornac JP, et al. Identification of a variety of *Staphylococcus* species by matrix-sssisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2010; 48: 941-945.

20. Wang J, Kliks MM, Qu WY, et al. Rapid determination of the geographical origin of honey based on protein fingerprinting and barcoding using MALDI TOF MS. J Agric Food Chem 2009; 57: 10081-10088.

4. Conclusions

A vast number of methods have been developed for the identification of genera, species, and strains of bacteria. These consist of culture methods (biochemical tests and chemical analysis of pure cultures), serological methods (ELISA and immunofluorescence assays) and genetic methods (nucleic acid hybridization and PCR techniques). The common methods for identifying bacteria in contaminated samples involve obtaining pure cultures first by streaking or sequential dilution. Microscopic observation and biochemical tests are followed by verification of the suspected identity with specific antibodies or molecular probes. PCR and commercial kits containing the antibodies or probes are sometimes used with mixed cultures to resolve quickly whether the DNA and proteins of a specific bacterium are present, and DNA microarrays can be used to test for numerous species of bacteria at one time. Nevertheless, if none of the probes or antibodies on hand correspond to any of the bacteria in the sample, then other tests with pure cultures will be necessary. Each of the methods has advantages for identification of certain species; however, none has yet accomplished all of the advantages of speed and accuracy with any serious weaknesses.

MALDI-TOF MS is a very rapid method for analyzing the proteins desorbed directly from whole cells. The whole-cell spectra produced by MALDI-TOF MS have taxonomically characteristic features that can be used to differentiate bacteria at the genus, species, and strain level, even though only a small number of proteins can be detected directly from whole cells. MALDI-TOF MS profile analysis results in similar family trees compared to classical methods like morphological and biochemical comparison or 16S ribosomal RNA sequencing. Because ribosomal proteins are highly abundant and appear very stable the observed protein pattern enables a direct view to the translated DNA sequence (Claydon *et al.*, 1996; Suh & Limbach, 2004).

Here a detailed study was performed using MALDI-TOF MS to analyse taxonomic relationships of human and veterinary important bacteria. For identification of clinical strains, a precondition was the establishment of high quality spectra libraries for the particular bacteria. The reference bacteria used for ‘in house’ database development were obtained from ATCC and NCTC (for *Campylobacter*, *Arcobacter* and *Helicobacter* species) these strains either were type strains of the species or matched to

a type strain in the Bruker reference database library. However, the *Gallibacterium* reference stains were obtained from Prof. Magne Bisgaard in Denmark.

The clinical and economic importance of *Campylobacter*, *Arcobacter*, and *Helicobacter* coupled to their taxonomic complexity has led to a wide range of phenotypic and genetic methods being developed to identify and differentiate them. Nevertheless, no method has yet accomplished to identify and differentiate all the relevant species in reasonable time frame and accuracy. However, by MALDI-TOF MS *C. jejuni*, *C. coli*, *C. lari*, *C. hyointestinalis* and *C. fetus* had unique and reproducible mass spectra and therefore the five species were easily differentiated. Furthermore, the closely related *Arcobacter* species *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*, and *Helicobacter* species, *H. pullorum* and *H. pamentensis* had unique and reproducible mass spectra. Thus, within minutes, the species from the three phenotypically similar genera were easily differentiated according to their mass signal patterns and therefore these signal patterns were used to build an 'in house' reference database.

A total of 144 clinical isolates (obtained from humans, environment and from different farm animals, mostly poultry) were correctly identified using the 'in house' MALDI/Biotyper reference database at species level and no differences in results were found associated with the source of the isolate. All strains were identified phenotypically and with PCR-RFLP. PCR-RFLP species identity results were 97.9 % concordant to MALDI-TOF MS results, because PCR-RFLP could not differentiate between *C. fetus* and *C. hyointestinalis* in three clinical isolates. In all these cases, a differentiation at species level with MALDI-TOF MS was possible. Correct identification can be obtained with MALDI/Biotyper even if the bacteria are stored at room temperature or at +4°C up to 9 days until being tested. However, the identification was affected when bacteria were grown on mCCD agar i.e. flatline spectra, hence, no identification possible with MALDI-TOF MS. Nevertheless, correct identification is possible when bacteria are grown on COS or CAM agar plates. Thus, MALDI-TOF MS fingerprinting is a fast and reliable method for the identification and differentiation of *Campylobacter*, *Arcobacter* and *Helicobacter* spp., with applications in clinical diagnostics.

MALDI-TOF MS revealed to be a rapid and reliable method even with the recently established genus *Gallibacterium*. This genus represents a phenotypically heterogeneous group, where identification and differentiation of species belonging to this genus is difficult with other means. *Gallibacterium anatis* is potentially pathogenic for poultry and is mainly associated with lesions in the reproductive tract, including the ovary (Gerlach, 1977; Bisgaard & Dam, 1981; Neubauer *et al.*, 2009). Disease associated with this microorganism is related to decreased egg production and occasionally an increase in mortality of birds. Therefore, 66 reference *Gallibacterium* strains were in-depth characterised by biochemical/physiological properties, genotypic methods and MALDI-TOF MS, and the results compared. According to the cluster analysis generated by the MALDI/Biotyper software, five different species were revealed which are the four type strains and an additional probable species. The MALDI-TOF MS results of all 66 strains demonstrated 87.9 % concordance with results based upon biochemical/physiological characterization. In addition, diversities outlined by MALDI-TOF MS were verified by sequencing the *rpoB* (n = 43), 16S rRNA (n = 28), *infB* (n = 14) and *recN* (n = 14) genes (multilocus sequence analysis, MLSA). However, discrepancies were observed between some of the genes sequenced. Reliable identification was possible when bacteria were stored at 4°C degree, even up to 8 days, this demonstrate that the MALDI/Biotyper species identification is reproducible even under different conditions. Nevertheless, if the strains were grown longer than 48 hrs at 37 °C under microaerobic atmosphere, poor identification results were obtained, due to changes in protein profile.

The *Gallibacterium* reference stains mass spectra were implemented into the Biotyper database for identification of clinical strains. MALDI-TOF MS identified 184 strains correctly in comparison to the results from *Gallibacterium*-specific PCR and AFLP. Different strains of *Gallibacterium* have highly different levels of virulence (Bisgaard, 1977; Gerlach, 1977; Mushin *et al.*, 1980). Therefore, MALDI/Biotyper based clustering was used to enable identification of possible specific clones of *G. anatis*. However, no different clonal lineages of *G. anatis* strains isolated from upper respiratory tract, reproductive tract, heart, liver and spleen within a bird was revealed. Hence, indicating that these *Gallibacterium* isolates are part of the normal flora of chickens and are an opportunist pathogen. Yet, different clonal lineages of *G. anatis* were observed between flocks, indicating each flock was infected with a single clone,

supporting the findings of genotypic methods. Therefore, we propose that the isolates that group together below 100-distance level in MALDI/Biotyper dendrogram are MALDI defined clones.

This thesis is a strong conformation of the ability of MALDI-TOF MS whole cell fingerprinting to be the next-generation of rapid molecular microbial identification system. The speed and minimal costs of sample preparation and measurement for this method makes it exceptionally well suited for routine and high-throughput use. This is extremely important in human and veterinary medicine where patients' fast recovery is in question. MALDI/Biotyper based clustering could be used as epidemiological method for evaluating bacterial disease in order to monitor the spread of an outbreak and to resolve where the infection started.

References

1. **Bisgaard, M. (1977).** Incidence of *Pasteurella haemolytica* in the respiratory tract of apparently healthy chickens and chickens with infectious bronchitis. Characterisation of 213 strains. *Avian Pathol* **6,** 285-292.

2. **Bisgaard, M. & Dam, A. (1981).** Salpingitis in poultry. II. Prevalence, bacteriology, and possible pathogenesis in egg-laying chickens. *Nordisk Vet Med* **33,** 81-89.

3. **Claydon, M. A., Davey, S. N., EdwardsJones, V. & Gordon, D. B. (1996).** The rapid identification of intact microorganisms using mass spectrometry. *Nat Biotechnol* **14,** 1584-1586.

4. **Gerlach, H. (1977).** Significance of *Pasteurella haemolytica* in poultry farms. *Prakt Tierarzt* **58,** 324-328.

5. **Mushin, R., Weisman, Y. & Singer, N. (1980).** *Pasteurella haemolytica* found in the respiratory-tract of fowl. *Avian Dis* **24,** 162-168.

6. **Neubauer, C., De Souza-Pilz, M., Bojesen, A. M., Bisgaard, M. & Hess, M. (2009).** Tissue distribution of haemolytic *Gallibacterium anatis* isolates in laying birds with reproductive disorders. *Avian Pathol* **38,** 1-7.

7. **Suh, M. J. & Limbach, P. A. (2004).** Investigation of methods suitable for the matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins from ribonucleoprotein complexes. *Eur J Mass Spectrom (Chichester, Eng)* **10,** 89-99.

5. Curriculum vitae

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Address: Heinrich Oeschl Gasse 19/1/1/3, 3430 Tulln, Austria

Nationality: Irish/Bosnian

Date of Birth: 11th October 1981

Marital Status: married (one daughter)

Employment

From 2007	Project assistant at the University of Veterinary Medicine, Vienna
2006-2007	Maternity leave
2005-2006	Project assistant at the University of Veterinary Medicine, Vienna

Education

2005–2010 **PhD (Dr. rer. nat.) in Genetics and Microbiology**

"Characterization of selected members of *Campylobacter*, *Arcobacter*, *Helicobacter* and *Gallibacterium* genera by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), with applications in clinical diagnostics"

Supervisor: Prof. Dr. Michael Hess

University of Vienna, Vienna, Austria

1999-2004 **Bachelor of Science Honours Biology**

Final year project in Molecular Evolution and Bioinformatics Laboratory **"**Co-evolution between different subunits of the CCT heat-shock protein: implications for Actin and Tubulin folding"

Supervisor: Dr. Mario A. Fares

Major in Honours Biology with Mathematics, Computer Science and Experimental Physics as minor

National University of Ireland, Maynooth, Co. Kildare, Ireland

1994-1999 **Leaving Certificate** 1999, **Junior Certificate** 1997
Hartstown Community School, Dublin 15, Ireland

Publications and Related Work

- **Merima Alispahic**, Karin Hummel, Delfina Jandreski-Cvetkovic, Katharina Nöbauer, Ebrahim Razzazi-Fazeli, Michael Hess and Claudia Hess. (2010) "Species Specific Identification and Differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by full-spectral MALDI-TOF MS analysis"**.** *Journal of Medical Microbiology* **59**, 295-301.

Oral Presentations

- Rapid identification and specification of selected *Campylobacter*, *Arcobacter* and *Helicobacter* by MALDI-TOF MS. 75. Fachgespräch über Geflügelkrankheiten. **Hannover, Germany**. November 2008.
- Rapid identification and speciation of selected *Campylobacter*, *Arcobacter* and *Helicobacter* by MALDI-TOF MS in comparison to PCR-RFLP. 6th Austrian Proteome Research Symposium. University of Technology, **Vienna, Austria**. September 2008.
- Expression of recombinant antigenic *Campylobacter jejuni* proteins using the Baculovirus system. Clinic seminar. University of Veterinary Medicine, **Vienna, Austria**. 2005.

Poster Presentation

- Rapid species identification and differentiation of *Arcobacter*, *Helicobacter* and *Campylobacter* by MALDI-TOF MS analysis and its clinical application. 20th ECCMID. **Vienna, Austria**. April 2010.

Workshops

- **"**Microbiology screening methods" and "Diagnostic tests". 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)**.Vienna, Austria**. April 2010.

- Certificate from AuPA – "From Study Design to Sample Preparation" and "Special Samples: Glycoproteins". University of Technology, **Vienna, Austria.** September 2007.

Professional Skills

MALDI-ToF MS

- Characterization of microorganisms by MALDI-TOF mass spectrometry and reference database development. Software: FlexContorl, FlexAnalises and BioTyper version 1.1 and 2.0

Molecular Biology Techniques

- Baculovirus Expression System and Insect Cell Culture (Sf9, Sf21 and Hi5), Protein purification (His-tag), ELISA development, PCR, Gelelectrophoreses, Cloning, SDS/PAGE, Western Blotting, Immunoprecipitation, Immunofluorescence

Bioinformatics Tools

- Sequence alignment; Clustal X, Phylogenetic tree reconstruction; MEGA, Visualisation and manipulation of the crystal structure; RASMOL, Analysis of selective constrains in protein–coding genes; PAML v3.12, and SWAPSC

Languages

- Fluent in English, Bosnian and conversational German

Computer Skills

- MSWord, Excel, PowerPoint, Databases, Reference Manager, Programming language JAVA