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Thermophilic *Campylobacter* in turkeys in Germany
Epidemiology, genotyping and antimicrobial sensitivity studies

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Abbreviations

asp	Aspartase gene
AT	ArrayTube
AVH	Avian vibrionic hepatitis
aw	Water activity
C	*Campylobacter*
CAT	Cefoperazone amphotericin teicoplan
CC	Clonal complex
CEB	Campylobacter enrichment broth
cfu	Colony forming unit
Ceu	Campylobacter enterochelin uptake
CLSI	Clinical and Laboratory Standards Institute
CSM	Charcoal selective medium
DNA	Deoxyribonucleic acid
E-test	Epsilometer test
EFSA	European Food Safety Authority
EU	European Union
*fla*A	Flagellin A gene
gln	Glutamine synthase gene
glt	Citrate synthetase gene
gly	Serine hydroxyl methyl transferase gene
gyr	Gyrase gene
hip	Hippurate gene
Ile	Isoleucine
ISO	International Standard Organization
MAMA-PCR	Mismatch amplification mutation assay polymerase chain reaction
*map*A	Outer membrane lipoprotein gene
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MLST	Multi locus sequencing typing
mPCR	Multiplex polymerase chain reaction
NaCl	Sodium chloride
O	*Ochrobactrum*
PCR-RFLP	PCR-restriction fragment length polymorphism
PEMS	Poult enteritis mortality syndrome
PFGE	Pulsed field gel electrophoresis
pgm	Phosphor glucomutase gene
pH	Measure of the activity of the (solvated) hydrogen ion
QRDR	Quinolone resistance determining region
*rec*A	Recognase A gene
ST	Sequence type
tet(O)	Tetracycline gene
Thr-86→Ile	Threonine-to-isoleucine mutation of amino acid 86 of the *gyr*A protein
tkt	Transketolase gene
unc	ATP synthase alpha subunit gene
VBNC	Viable but non culturable form
VLO	Vibrio like organism

Dedecation

I dedicate this work to the memory of my mother and father who so nobly gave their best for me.

****

I dedicate this work to my family.

****

I dedicate this work to my supervisors.

Hosny El-Adawy

Introduction

Campylobacter has emerged as food born pathogen with increasing concern replaced Salmonellae especially in developed countries (EFSA, 2012a).

In poultry production, *Campylobacter* contamination is undeniably a major food safety issue. The majority of research on *Campylobacter* infections focused on broilers, however, turkeys have also been identified as an important reservoir for *Campylobacter*. Therefore, there is a need to focus attention on mechanisms of transmission and epidemiology under commercial operations coupled with molecular tracking of involved strains. Detection and differentiation of thermophilic *Campylobacter* by conventional culture methods and biochemical identification is time and cost intensive. In contrast, multiplex PCR used for detection of thermophilic *Campylobacter* is characterized by speed, reliability and cost efficiency (Denis et al., 1999; Denis et al., 2001; Lund et al., 2003; Perko-Mäkelä et al., 2009). The method can be applied for direct detection of pathogen DNA extracted from clinical samples (Lund et al., 2003; El-Adawy et al., 2012a).
Genetic diversity among thermophilic *Campylobacter* spp. may enable its survival in the environment (Parkhill et al., 2000). Molecular typing tools have improved our understanding of the epidemiology of bacterial food-borne pathogens (Wassenaar and Newell, 2000). Genetic profiles are different according the used molecular typing methods (El-Adawy et al., 2013).

The emergence of antibiotic resistance in *Campylobacter* in particular to those antibiotics used to treat human illness has been increased (EFSA, 2012b). Campylobacters isolated from turkeys have a high level of resistance to antibiotics (Ge et al., 2003).

For clinical therapy of campylobacteriosis, a macrolide is considered to be the drug of choice, but fluoroquinolons and tetracycline are also frequently applied (Luangtongkum et al., 2009). These drugs are also used in poultry production and might result in the development of antibiotic-resistant bacteria.

Molecular techniques to study antibiotics resistance mechanisms are useful tools for monitoring programs (Zirnstein et al., 1999; Vacher et al., 2003; Mazi et al., 2008).

Beside *Campylobacter* several other bacteria could be isolated from turkey house and environemt. During the present investigations *Ochrobactrum* species were isolated.

The aim of this work is to investigate several aspects of the epidemiology and antimicrobial resistance of *Campylobacter* in turkey production. These include:

1. Development of modified mPCR for detection of DNA of thermophilic campylobacters.
2. Investigation of genetic diversity and dynamics of *C. jejuni* among a fattening turkey flock.
4. Determination of antimicrobial sensitivities among *C. jejuni* isolated from turkey farms in Germany.
5. Molecular detection of resistance of *C. jejuni* gens to clinically relavent antibiotics namely erythromycin, ciprofloxacin and tetracycline.
6. Study of the phenotypic and genotypic characters, and antimicrobial sensitivity of two *Ochrobactrum* spp. isolated from turkey flock.

C H A P T E R 1

Review of Literature

Review of literature

1. *Campylobacter*: Organism

1.1. Historical aspects

A member of the genus *Campylobacter* was first recognised by Theodore Escherich at the end of the 19th century as spiral bacteria in the colon content of children, who died, of what he called 'cholera infantum' (Vandamme, 2000). From the beginning of the twentieth century these "vibrio-like bacteria" were considered as veterinary pathogens. *Campylobacter* was identified for the first time in 1906 in the uterine mucus of pregnant sheep. McFadyean and Stockman in 1913 isolated these microorganisms from aborted bovine fetuses, the bacterium named *Vibrio* (now *Campylobacter*7*fetus* (Skirrow, 2006). Later in 1927, Smith and Orcutt named a group of bacteria, isolated from the faeces of cattle with diarrhea, as *Vibrio jejuni* (now *Campylobacter jejuni*7*(*Jones et al., 1931). In 1944, Doyle isolated a different *Vibrio* species from faeces of pigs with diarrhea and classified them as *Vibrio coli* (Vandamme, 2000; Vandamme et al., 2010). *Campylobacter* were isolated from humans for the first time in conjunction with a milk-borne food poisoning outbreak in the United States in 1938 (Levy, 1946). In the late 1950s, *Campylobacter* were isolated from blood samples of children with diarrhoea (King, 1957). In 1963, based on the DNA content, the organism was renamed by Sebald and Veron as *Campylobacter* (On, 2001).

1.2. Taxonomy and structure

The taxonomic structure of the genus *Campylobacter* has experienced extensive changes and even some parts of the current genus taxonomy remain a matter of controversy and require further investigation (Vandamme, 2000; On, 2001; Vandamme and On, 2001; Debruyne et al., 2008). *Campylobacter* species belong to the epsilon class of *Proteobacteria* (Euzéby, 1997). The class *Epsilonproteobacteria* was described on the basis of phylogenetic analysis of 16S rRNA sequences; the class contains the order *Campylobacterales*. The order contains the families *Campylobacteraceae*, *Helicobacteraceae*, and *"Nautiliaceae"*. The genera *Campylobacter*, *Arcobacter*, *Sulfurospirillum*, and the genetically misclassified species *Bacteroides ureolyticus*, constitute the family *Campylobacteraceae* (Vandamme, 2000). At present, the genus *Campylobacter* contains 17 species, four of them have been further divided into eight subspecies (Vandamme and On, 2001) (Table1). The species *C. concisus, C. curvus, C. hominis, C. sputorum, C. rectus, C. showae* and *C. gracilis* are phylogenetically closely related.

Within the genus *Campylobacter*, the group of the thermophilic (or more accurately, thermotolerant) campylobacters (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*) forms a distinct subcluster including also *C. helveticus* (Vandamme, 2000).

Table 1. *Campylobacter* taxonomy

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Epsilonproteobacteria*
Order	*Campylobacterales*
Family	*Campylobacteraceae*
Genus	*Campylobacter*
Species	*C. coli, C. concisus, C. curvus, C. faecalis, C. fetus , C. gracilis, C. helveticus, C. hominis, C. hyointestinalis , C. jejuni , C. jejuni-like bacterium, C. lanienae , C. lari, C. mucosalis, C. mucosalis-like bacterium, C. rectus, C. showae, C. sputorum , C. upsaliensis*

1.3. General characteristics

The *Campylobacter* is derived from the Greek words "*campylos*", which means "curved" and I*baktron*", which means "rod". Campylobacters are Gram negative, non-spore-forming and slender spirally curved rods (0.2–0.8μm×0.5–5μm) (Vandamme, 2000). The majority of the species have a corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell which can be observed by phase contrast or darkfield microscopy. The exceptions are *C. gracilis* which is non-motile and *C. showae* which has multiple flagella (Debruyne et al., 2008). Campylobacters are relatively slow-growing, fastidious bacteria. They are microaerophilic (Debruyne et al., 2008). The thermophilic species are characterized by their ability to grow between 37^{o}C and 42°C with an optimum temperature of 41.5˚C (Levin, 2007) and their inability to grow at 25°C (absence of cold shock protein genes, which play a role in low-temperature adaptation). Optimal growth of *C. jejuni* occurs with water activity (*a*w) 0.997 (approximately 0.5% w/v NaCl). *Campylobacter* survives for more than 4h at 27˚C and 60–62% relative humidity on some common clean or soiled food contact surfaces. *Campylobacter* will not survive below a pH of 4.9 and above pH 9.0 and grow optimally at pH 6.5–7.5 (De Cesare et al., 2003). *Campylobacter* spp. have small genomes (*C. jejuni* and *C. coli* 1.7 to 1.8 Mb), which have only about 36% of the size of the *E. coli* chromosome (Parkhill et al., 2000). It is well known that *C. jejuni* comprises an extremely diverse population with a broad spectrum of subtypes (Wassenaar and Newell, 2000).

The *Campylobacter* flagellum is composed of two highly homologous flagellins, *fla*A which is the major one and *fla*B as the minor one (Guerry, 2007). The *fla*A seems to be essential for the invasion of epithelial cells, since it has been reported that a mutation in this gene leads to a truncated flagellar filament composed of *fla*B with a severe reduction in its motility.

However, a mutation in *fla*B appears to have no significance compared with a structurally

normal flagellum (Guerry, 2007). The *fla*A gene is responsible for the expression of adherence, colonization of the intestinal tract and invasion of the host cells (Jain et al., 2008).

1.4. Viable non culturable *Campylobacter* (VBNC)

Under unfavorable growth conditions, *Campylobacter* has the ability to form viable but non-culturable cells (VBNC) (Portner et al., 2007). Exposure to acidic conditions may trigger a conversion of *C. jejuni* into a VBNC form that allows the organism to restore culture ability when favourable conditions are encountered (Chaveerach et al., 2003; Klancnik et al., 2009). The key test that determines whether such cells are dead, or alive but in dormant state, is the viability count (Oliver, 2005). Cell viability or cellular respiration is proven by assessing the maintenance of cellular integrity using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Boucher et al., 1994) or double staining with CTC-DAPI (4'6'-diamidino-2-phenylindole) (Chaveerach et al., 2003) or fluorescent dye Syto9 and propidium iodide (Klancnik et al., 2009). The resuscitation of VBNC *C. jejuni* cells has been demonstrated after passage through embryonated eggs which suggests that they have maintained their pathogenicity and could pose a threat to public health (Cappelier et al., 1999). These VBNC cells would not be detected by conventional culture techniques and therefore could be considered as a public health risk.

1.5. Isolation of *Campylobacter*

Isolation of *Campylobacter* can be performed by bacteriological methods and generally requires 4 days to give a negative result and 6–7 days to confirm a positive result in identification (Corry et al., 1995). Commonly used selective media with blood components for *Campylobacter* include Skirrow agar (Skirrow, 1977) and Preston agar (Bolton and Robertson, 1982), while selective media with charcoal include Cefoperazone Amphotericin Teicoplan (CAT) agar (Aspinall et al., 1993), modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) (Hutchinson and Bolton, 1984) and Karmali agar or Charcoal Selective Medium (CSM) (Karmali et al., 1986). Brilliance CampyCount agar is a novel, highly selective and chromogenic medium specifically designed for accurate, specific and easy-to-read identification and enumeration of *C. jejuni* and *C. coli* (ISO, 2006).

The selectivity of the medium is determined by the use of antibiotics. Cephalosporins are often used in combination with other antibiotics such as trimethoprim, vancomycin, amphotericin, rifampicin (Corry et al., 1995). The fungistatic agent cycloheximide or amphothericin are added (Bolton et al., 1988).

Pre-enrichment media commonly used are Exeter broth, Bolton broth, Preston broth, Campylobacter Enrichment Broth (CEB) and Park & Sanders broth (Baylis et al., 2000). Microaerobic atmospheres of 5–10% O_2, 5–10% CO_2 are required for optimal growth (Corry et al., 1995; Vandamme, 2000). Media may be incubated at 37°C or 42°C, but it is common practice for thermophilic campylobacters to incubate at 42°C to minimise growth of contaminants (Bolton et al., 1988).

The method (direct plating vs. enrichment), type of sample (caecal content, fresh droppings, litter), sampling time, type of rearing (conventional, free-range, and organic) age and breed of birds can influence the isolation and /or the detection of *Campylobacter* spp. (Jørgensen et al., 2002).

1.6. Detection of *Campylobacter*

As thermophilic campylobacters are difficult to cultivate by conventional cultural methods, several alternative and rapid methods have been developed for detecting and confirming *Campylobacter* spp., e.g. those that include fluorescence in situ hybridization (Lehtola et al., 2006), latexagglutination (Wilma et al., 1992), enzyme immunoassay (Endtz et al., 2000), and PCR-based methods (Lund et al., 2003; El-Adawy et al., 2012a). PCR performance on direct testing of field samples seems to be reduced drastically due to PCR inhibitors that may be present in faecal material (Sahin et al., 2003b). Another drawback of PCR methods is that they are unable to discriminate between viable and non-viable *Campylobacter* cells, which may be essential for some epidemiological studies (Sahin et al., 2003b).

Campylobacter colonies are greyish and slightly pink with a metallic sheen on blood-based Preston agars, while on charcoal-based media the colonies are greyish to white with a metallic sheen and on Brilliance CampyCount Agar are distinct dark red colonies against a transparent medium (Figure 1).

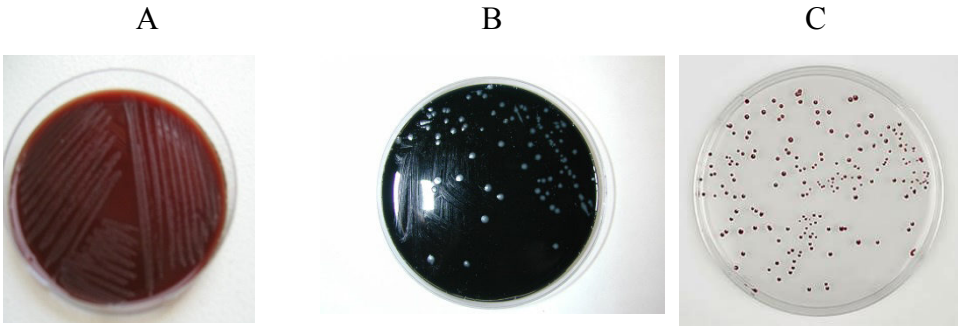

Figure 1 *C. jejuni* colonies on blood-based Preston agar (A), mCCDA (B) and Brilliance CampyCount Agar (C)

1.7. Phenotyping of *Campylobacter*

Microscopic appearance, motility and biochemical reactions amend the procedure (Eberle and Kiess, 2012). The biochemical characteristics of each *Campylobacter* species have been summarized by Kaplan and Weissfeld, 1994 (Table 2).

Table 2. Differentiation characters of thermophilic *Campylobacter* spp. (Kaplan and Weissfeld, 1994)

Criteria	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
Catalase test	+	+	+	-
Oxidase test	+	+	+	+
Urease test	-	-	-	-
Nitrate reduction	+	+	+	+
Selenite reduction	+	+	+	+
Fumerate reduction	+	+	+	+
Voges–Proskauer reaction	-	-	-	-
CHO fermentation	-	-	-	-
Naladixic acid	Sensitive	Sensitive	Resistant/Sensitive	Sensitive
Cephalothin	Resistant	Resistant	Resistant	Sensitive
Metronidazole	M	+	+	M
Hippurate hydrolysis	+	-	-	-
Indoxyl acetate hydrolysis	+	+	-	+
%&S/TSI	-	F	-	-
25°C	-	-	-	-
42°C	+	+	+	M
1% glycine	M	+	+	+
2.0% NaCl	-	-	M	-
2.0% ox-bile	M	M	M	+
Growth on minimal medium	-	+	-	-
Sodium fluoride	+	+	+	-
$KMnO_{\$}$	+	+	F	F
0.02% safranin	+	+	+	F

F - 14–50% strains positive; M - 60–93% strains positive.

1.8. Molecular identification of *Campylobacter*

Differentiation of *Campylobacter* by PCR has been described previously (Denis et al., 1999; Sahin et al., 2003b; El-Adawy et al., 2012a). Other molecular detection and identification methods for *Campylobacter* spp. have been reviewed by Sahin et al. (2003b).

1.9. Genotyping of *Campylobacter*

1.9.1. Flagellin typing using Restriction fragment length polymorphism (*fla*-RFLP)

The *fla*A and *fla*B are suitable for RFLP analysis of PCR products (Meinersmann et al., 1997). The *fla* typing procedures have been developed and evaluated (Wassenaar and Newell, 2000), however there are considerable variation in the PCR-RFLP procedures as to DNA

preparation techniques, primer design, annealing temperatures and restriction enzymes used (El-Adawy et al., 2013). These not allow a direct comparison of results obtained in different laboratories (Nachamkin et al., 1993). The enzymes *Alu*I, *Dde*I, *Eco*RI, *Hin*fI, *Pst*I and *Sau3*AI are currently used in various combinations (http://campynet.vetinst.dk/Fla.htm).

1.9.2. Pulsed field gel electrophoresis (PFGE)

Digestion of bacterial chromosomes by restriction enzymes that cleave the DNA infrequently has proved to be a useful typing technique for many bacteria (Wassenaar and Newell, 2000). Differences in electrophoretic conditions can even lead to apparent differences in the profiles obtained for the same DNA preparation. Different restriction enzymes were used to digest the chromosomal DNA, however, satisfactory results have been obtained using *Sma*I, *Sal*I, *Kpn*I, *Apa*I, and *Bss*HII. Using of more than one enzyme significantly increases the discriminatory power (Wassenaar and Newell, 2000).

1.9.3. DNA sequencing

Direct DNA sequencing is becoming increasingly automated and consequently is a reasonable alternative typing method (Wassenaar and Newell, 2000). The advantage of sequence analysis is that it is highly reproducible and the results are easy to interpret. However, the complex data and superb discriminatory power make interpretation highly dependent on computerized comparison programs and the parameters set by the software packages used. Sequence analysis has been applied to the flagellin locus in several studies (Wassenaar and Newell, 2000).

1.9.4. Multilocus sequencing typing (MLST)

Multilocus sequence typing (MLST) has been developed to overcome the problems of comparing results of typing schemes between laboratories (Maiden et al., 1998; Dingle et al., 2001). MLST assigns the alleles of the 7 house keeping genes *asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt*, and *unc*A. In MLST, each sequence of a locus is assigned by an allele number in order of its discovery. The alleles are combined into an allelic profile and resulting in a sequence type and clonal complex (Maiden et al., 1998). Putative phylogenetic relationships among the STs and CCs were presented using a minimum spanning tree (http://pubmlst.org/campylobacter). The sequence data can be easily compared among laboratories (Dingle et al., 2001). The discriminatory power and reproducibility of MLST is high. Multilocus sequence typing can

also detect mixed cultures, genetic exchange, and recombination between *Campylobacter* spp. (Miller et al., 2005). Disadvantages of MLST are their complexity and high price (Lévesque et al., 2008).

1.9.5. Microarray based assay

Use of microarrays was confirmed to be a powerful diagnostic tool for monitoring emerging *Campylobacter* pathotypes as well as for epidemiological, environmental, and phylogenetic studies including the evaluation of genome plasticity (Marotta et al., 2013). DNA microarray technology with its ability to detect thousands of distinct DNA sequences simultaneously has been recognized as a potentially valuable tool for high-throughput, quantitative and detailed studies of microbial communities with a great saving of time (Ehricht et al., 2006; Taboada et al., 2007; Marotta et al., 2013). The ArrayTube™ (AT^{TM}) system is a less expensive platform and characterizes *C. jejuni* isolates by specific hybridization patterns based on a limited number of gene loci. A further advantage of the AT^{TM} system is the use of enzyme-catalysed precipitation staining rather than fluorescence detection which allows a measurement by a simple transmission technique (Ehricht et al., 2006).

2. Thermophilic *Campylobacter* in poultry

Commercial poultry such as broilers, layers, turkeys, and ducks as well as free living birds are considered to be reservoirs of thermophilic *Campylobacter* with variable prevalence. *Campylobacter* spp. are rarely detected in commercial broiler flocks under the age of 2 - 3 weeks (Newell and Fearnley, 2003). The explanation for this phenomenon is unclear, however, it is possible that an inhibitory effect as *Campylobacter*-specific maternal antibodies in young chicks acts (Newell and Fearnley, 2003; Sahin et al., 2003b) or the presence of unique microbial flora in the intestinal tract especially in the caecum of these young chicks prevents early colonization (Sahin et al., 2002). The prevalence of *Campylobacter* spp. in broilers increases with the age of the birds (Newell and Fearnley, 2003; Luangtongkum et al., 2006). Galliformes showed the highest prevalence rate followed by Anseriformes and Columbiformes (Yogasundram et al., 1989). Commercial ducks and geese are also frequently affected (Aydin et al., 2001; Nonga and Muhairwa, 2009). Turkeys have been identified as an important reservoir for thermophilic *Campylobacter* (Hafez et al., 2001; Luangtongkum et al., 2006; Perko-Mäkelä et al., 2009; Perko-Mäkelä et al., 2011).

In EU, the colonization of commercial broiler flocks with *Campylobacter* was variable (EFSA, 2012a). In the USA and Canada, the prevalence of *Campylobacter* in commercial broiler production was different (Newell and Fearnley, 2003; Luangtongkum et al., 2006). Likewise, variation in the prevalence of thermophilic *Campylobacter* among commercial broiler flocks was also observed in other regions of the world e.g. in Africa (Nonga and Muhairwa, 2009) and Asia (Sasaki et al., 2011).

The seasonal variation may correlate directly with other sources of contamination within the farm environment that are temperatures related to environmental such as migratory birds, rodents, and insects (Wallace et al., 1997). The highest rates were found in autumn and in spring (Sulonen et al., 2007). *C. jejuni* was the predominant species in commercial broilers, *C. coli* and *C. lari* are less common (Denis et al., 2001; Luangtongkum et al., 2006).

2.1. Transmisson

2.1.1. Horizontal transmission

Campylobacter transmission from a contaminated flock to the following flock seems to be not very important as *Campylobacter* is actually sensitive to detergents and disinfectants as well as dry conditions found in the poultry house during the service period (Evans and Sayers, 2000). *Campylobacter* spp. usually have not been isolated from fresh litter and feed samples before the flock had been colonized with *Campylobacter* (Sahin et al., 2002; Newell and Fearnley, 2003).

Contaminated and untreated drinking water plays an important role in the faecal–oral spread through the flock (Sahin et al., 2002; Newell and Fearnley, 2003; Bull et al., 2006; El-Adawy et al., 2012a).

The presence of domestic livestock including cattle, sheep, pigs and multiple avian specieses as well as domestic pets such as dogs and cats on farms has been associated with an increased risk of *Campylobacter* infection in broiler flocks (Sahin et al., 2002; Newell and Fearnley, 2003).

Insects including flies, darkling beetles, cockroaches, and mealworms in and around poultry houses can serve as vectors for *Campylobacter* spp. (Sahin et al., 2002; Newell and Fearnley, 2003).

Personnel and farm equipment such as trucks, forklifts, pallets, crates, and footwear have also been identified as potential sources of *Campylobacter* infection of poultry (Newell and Fearnley, 2003). Partial depopulation/thinning of broiler flocks have been implicated as a

potential risk factor for *Campylobacter* colonization of the remains the birds of these flocks (Patriarchi et al., 2011).

2.1.2. Vertical transmission

Campylobacter can be present in the poultry reproductive system. Nevertheless, several authors dismiss the assumption that vertical transmission is a major source of pathogen transmission (Sahin et al., 2003a; Sahin et al., 2003c). Isolation of *Campylobacter* spp. from broiler flocks before 2 or 3 weeks of age was hardly accomplished, even though the chicks were hatched from eggs obtained from infected parent flocks (Sahin et al., 2002; Newell and Fearnley, 2003).

Although other studies suggest that vertical transmission of *Campylobacter* spp. via the egg is considered unlikely (Sahin et al., 2002; Newell and Fearnley, 2003). *Campylobacter* cannot survive on egg shells and is unable to penetrate eggs under natural conditions (Perko-Mäkelä et al., 2009).

The vertical transmission of *Campylobacter* spp. from breeder flocks to broiler flocks through the egg may occur (Sahin et al., 2002; Newell and Fearnley, 2003).

2.2. Pathology of *Campylobacter* infection in poultry

Pathogenic isolates induced diarrhoea of short duration in newly hatched chicks (Sanyal et al., 1984). However, generally colonized birds usually showed no clinical signs; even when young birds are exposed to high doses under experimental conditions (Newell, 2002). Sometime symptoms of enteritis and hepatitis or excessive mortality could be observed in very young chicks (Corry and Atabay 2001).

The lesions appeared during *Campylobacter* infection in chicks comprise distention of the jejunum with accumulation of mucus and fluid (Sanyal et al., 1984) or disseminated areas of haemorrhage (Welkos, 1984). Infection of chicks by contact at the time of hatch resulted in focal hepatic necrosis (Clark and Bueschkens, 1988).

2.2.1. Avian vibrionic hepatitis

Avian vibrionic hepatitis (AVH) is defined as chronic debilitating condition of commercial laying flocks. It is characterised by the presence of greyish-white focal lesions in the liver, usually 1-2 mm in size. The presence of *Campylobacter* spp. in the liver alone is not sufficient to cause vibrionic hepatitis, but a further predisposing factor, possibly within the host, is

required (Jennings et al., 2010). A curved vibrio-like organism (VLO), sensitive to tetracycline and furazolidone, was accepted to be the causal agent (Winterfield et al., 1958). This organism was extensively investigated during the late 1950s and 1960s (Winterfield and Sevoian, 1957; Gerlach and Gylstorff, 1967).

2.2.2. Poult enteritis mortality syndrome

Poult enteritis mortality syndrome (PEMS) is a group of multifactorial, transmissible, infectious diseases of young turkeys less than six weeks of age. It is characterized by clinical signs of enteritis, moderate to marked growth depression (stunting), retarded development (runting), impaired feed utilisation, and secondary nutritional deficiencies (Barnes et al., 2000). Turkey flocks have a mortality of 9% during days 7-28 or a mortality of 1% per day over three consecutive days. Subclinical forms of the syndrome are responsible for growth depressions of 10-15%. Immune dysfunction generally occurs, which increases susceptibility of the flock to other infectious diseases (Barnes et al., 2000). *C. jejuni* frequently is encountered during the observation period (Lam et al., 1992; Barnes et al., 2000).

3. Public health significance of *Campylobacter*

Acorrding to EFSA, (2012a) the number of reported human campylobacteriosis in the EU due to handling raw poultry or consuming undercooked poultry increased by 6.7 % in 2010 compared to 2009 (Figure 2).

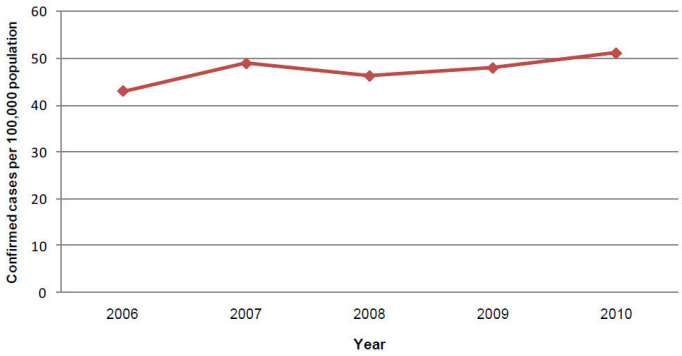

Figure 2. Campylobacteriosis confirmed cases in the EU (EFSA, 2012a).

Chicken and turkey meat appeared to be a common source of *C. jejuni* (Zhao et al., 2001). The highest number and notifications of campylobacteriosis cases in humans is reported during the summer months (Figure 3).

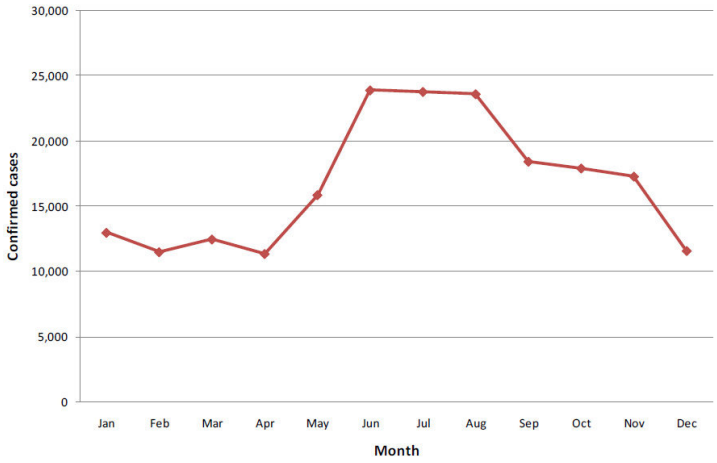

Figure 3 Seasonal variation of campylobacteriosis cases in humans in 2010 (EFSA, 2012a)

4. Antibiotic resistance in *Campylobacter*

The worldwide increase of antibiotics usage as an integral part of the poultry production has led to the problem of development of bacterial antibiotic resistance (Engberg et al., 2006).

There is partial association between the veterinary use of antibiotics and the emergence of resistant strains of *Campylobacter* related to human enteritis (Desmonts et al., 2004; Luangtongkum et al., 2006). *C. jejuni* isolated from turkeys had significantly higher resistance than broilers or layers (Ge et al., 2003).

The levels of resistance of *C. jejuni* from poultry to antibiotics were varied considerably in EU (Desmonts et al., 2004).

4.1. Antimicrobial susceptibility testing methods

Several methods for antimicrobial susceptibility testing of *Campylobacter* can be used (Nachamkin et al., 2000).

The agar diffusion test (disk diffusion) is widely used, easy to perform, convenient, inexpensive and can provide reproducible results especially when several antimicrobial agents need to be tested, but it requires a high level of standardization and quality control (Potz et al., 2004).

The E-test can provide quantitative minimal inhibitory concentration (MIC) values although its cost is much higher than that of the disk diffusion method (Ge et al., 2002).

The dilution test (agar dilution and broth microdilution) has been considered a standard method for thermophilic *Campylobacter* (CLSI, 2010). Although this method is very reliable, highly reproducible and provides quantitative MIC values, it is labour-intensive, time

consuming and quite expensive especially when compared to the disk diffusion method (Gaudreau and Gilbert, 1997).

Since currently there are no internationally accepted standard resistance breakpoints specific for *Campylobacter* available, the resistance breakpoints of enteric bacteria in the family *Enterobacteriaceae* have been used to determine antimicrobial resistance of thermophilic *Campylobacter* (Ge et al., 2002; Luber et al., 2003; El-Adawy et al., 2012c).

4.2. Mechanisms of antimicrobial resistance

4.2.1. Fluoroquinolone resistance

Two mechanisms are known which confered fluoroquinolones resistance to *Campylobacter;* point mutations in the quinolone-resistance determining region (QRDR) of *gyr*A and the multidrug efflux pump CmeABC. The most commonly found mutation in *C. jejuni* highly resistant to ciprofloxacin is the Thr86-Ile mutation (Zirnstein et al., 1999).

4.2.2. Tetracycline resistance

The mechanism of tetracycline resistance in *Campylobacter* is primarily associated with the *tet*(O) gene, which encodes a ribosomal protection protein designated as Tet(O) (Connell et al., 2003). The multidrug efflux pump CmeABC has been implicated in intrinsic and acquired tetracycline resistance. Addition of the pump inhibitor PAβN reduced tetracycline resistance less than two fold (Gibreel et al., 2007).

4.2.3. Macrolide resistance

The mechanism of high level macrolide resistance in *Campylobacter* associated with an alteration of the target site on the 23S rRNA gene or mutations at positions 2074 and 2075 (Aarestrup and Engberg, 2001; Vacher et al., 2003; Gibreel and Taylor, 2006). The involvement of an efflux system in the macrolide resistance of *C. jejuni* and *C. coli* using the efflux pump inhibitor β-naphtylamide (PAβN) has been demonstrated (Gibreel et al., 2007). In *Campylobacter* strains with low-level erythromycin resistance (MICs 8–16 µg/ml), no mutations have been detected (Payot et al., 2004).

4.2.4. Aminoglycosides

The most common enzyme in *Campylobacter* spp. that involves kanamycin resistance and structurally related antibiotics such as neomycin resistance is the 3'-aminoglycoside

phosphotransferase type III [APH-III], which is encoded by the *aph*A-3 gene. Other genes such as *aph*A-7 and *aph*A-1 are also associated with a high-level of kanamycin resistance in *Campylobacter* and *Campylobacter*-like organisms, respectively (Gibreel et al., 2004).

4.2.5. Beta-lactam resistance

The main resistance mechanism for *C. jejuni* and *C. coli* isolates to beta-lactam antimicrobial agents seems to be associated with the production of beta-lactamases, which break the beta-lactam ring, hydrolyse the structural lactam ring and influence efflux pumps (Aarestrup and Engberg, 2001). Other mechanisms of resistance such as alteration of penicillin-binding proteins or decreased permeability of the drug through modification of porins could be involved in ampicillin resistance in *Campylobacter* spp. (Aarestrup and Engberg, 2001).

4.2.6. Sulphonamide resistance

Sulphonamide resistance in *C. jejuni* is associated with the mutational substitution of four amino acid residues in dihydro-pteroate synthetase (DHPS) resulting in a reduced affinity for sulphonamides (Gibreel and Sköld, 1999).

4.2.7. Trimethoprim resistance

Trimethoprim acts by binding to and inhibiting the activity of dihydrofolate. Resistance is due to the acquisition of horizontally transferred reductase *dfr* genes that are not inhibited by trimethoprim. In *Campylobacter* two different genes (*dfr*1and *dfr*9) have been found to mediate resistance (Gibreel and Sköld, 1999).

5. *Ochrobactrum*

The genus *Ochrobactrum* was first associated in 1988 with the organisms formerly known as CDC group Vd (Holmes et al., 1988). These are bacteria non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, Gram negative rods. Molecular taxonomy places *Ochrobactrum* in the alpha-subgroup of proteobacteria, closely related to the genus *Brucella* belonging to the family *Brucellaceae* (Velasco et al., 1998; Lebuhn et al., 2000).
The *rec*A sequence analysis provides a reliable molecular subtyping tool to study the phylogeny of the *Ochrobactrum* taxon at both, the inter- and intra-species level (Scholz et al., 2006; ElAdawy et al., 2012b). The *rec*A analysis significantly describes phylogenetic position of atypical *O. anthropi* isolates within the *Ochrobactrum–Brucella* group (Scholz et al.,

2006). *Ochrobactrum* species have been described as free-living pathogens. They are characterized by an exceptional level of genomic diversity that could be related to the adaptability of *Ochrobactrum* species to various ecological niches (Teyssier et al., 2005). The genus *Ochrobactrum* comprises 16 species (Kämpfer et al., 2010). Only *O. gallinifaecis* was isolated from fecal matter collected from chicken farms (Kämpfer et al., 2003).

O. anthropi has been isolated from various clinical specimens and is recognized as an opportunistic pathogen of zoonotic importance (Gill et al., 1997; Stiakaki et al., 2002). It was described as Gram negative bacterium with high resistance particular to all beta-lactam antibiotics (Nadjar et al., 2001; ElAdawy et al., 2012b).

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C H A P T E R 2

Epidemiology of *Campylobacter* in turkey

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Elucidation of colonization time and prevalence of thermophilic *Campylobacter* species during turkey rearing using multiplex polymerase chain reaction

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1. Summary

Two turkey flocks (male and female) and the environment of their house were investigated for the presence of thermophilic *Campylobacter*. Sample DNA was extracted directly from fecal material and environmental samples. Bacterial identification was done using a modified *Campylobacter* species specific multiplex PCR. The times needed for colonization and prevalence in male and female turkeys were determined independently. All environmental samples collected before restocking were negative in the PCR analysis, showing a good hygiene and biosecurity system. The first positive PCR results were obtained in drinking water samples at 6 d of age. Colonization occurred between the second and third week of age, starting in female birds and then followed by the males. *Campylobacter jejuni* was detected by multiplex PCR at first; later on, *Campylobacter coli* and mixtures of both were seen. After the 9 wk of age, the colonization of the flocks was completed. Great attention should be given to drinking water as a supposed source of *Campylobacter* contamination. Multiplex PCR proved to be a rapid, sensitive, and cheap tool for the diagnosis of *Campylobacter* contamination.

2. Introduction

Thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*) are the causative agents of acute food-borne bacterial enteritis in humans. In 2008, the number of human *Campylobacter* enteritis cases in Germany was higher than that of *Salmonella* infections (Robert-Koch-Institut, 2010). Poultry remains the most important reservoir and source for human infections (van Vliet and Ketley, 2001). The normal body temperature of fowl is 41 to 42°C, which coincides with the optimum growth temperature of thermophilic *Campylobacter* species (Corry and Atabay, 2001; Engvall, 2001; Petersen et al., 2001; Hariharan et al., 2004). An European food safety authority report (EFSA, 2010) demonstrated that approximately

80% of chicken carcasses of the European Union market are contaminated with *Campylobacter*, whereas *Salmonella* was detected in only 16% of chicken carcasses in 22 EU countries. The status of *Campylobacter* contamination during the various stages of turkey meat production is under investigated, although the consumption of turkey meat in Germany has increased during the last years and reached 6.5 kg per person in 2005 (Atanassova et al., 2007). Consequently, it is of great importance to identify and assess the potential risks of infection along the production chain of turkey meat products (Atanassova et al., 2007). Previous studies showed that chicks were apparently not infected at the hatch, indicating that the young chicks became infected at the farm through the introduction of the organism via biotic and abiotic sources that may include drinking water, feed, litter, worker boots, wild animals, insects, and so on (Gregory et al., 1997; Wesley and Muraoka, 2011). In recent years, it has become evident that biofilms in drinking water pipe networks can become transient or long-term habitats for hygienically relevant microorganisms, including *Campylobacter*. In these habitats, *Campylobacter* can survive for days to weeks or even longer (Wingender and Flemming, 2011). Drinking water was found to be the primary source of flock contamination (Bull et al., 2006; Sasaki et al., 2011). Shanker et al. (1990) succeeded to infect broilers with artificially contaminated drinking water. In contrast, Pokamunski et al. (1986) and Patriarchi et al. (2009) found no *Campylobacter* in water samples from all farms they surveyed. No differences in the colonization and prevalence were found between male and female turkey flocks over time (Cox et al., 2000).
Detection and differentiation of thermophilic *Campylobacter* from clinical samples by conventional culture methods and biochemical identification tests are time and cost intensive. The PCR was introduced to improve sensitivity (Gonzalez et al., 1997; Linton et al., 1997; Lawson et al., 1998; Denis et al., 1999; Denis et al., 2001; Lund et al., 2004). It is characterized by speed, reliability, and cost efficiency and can be applied for direct detection of pathogens from clinical and food samples (Wang et al., 2002; Lund et al., 2003; Khan and Edge, 2007; Perko-Mäkelä et al., 2009). Efficient extraction of *Campylobacter* DNA directly from different clinical matrices is a prerequisite for successful detection. Use of commercial extraction kits guarantees the preservation of highly purified DNA (Lund et al., 2003; Gioffré et al., 2004; Scupham, 2007).

The aim of this study was to determine the kinetics of *Campylobacter* colonization and prevalence of *Campylobacter* in groups of male and female turkeys using a modified multiplex PCR (mPCR) assay. Possible ways of transmission of thermophilic campylobacters were investigated to promote the development of effective programs for the reduction of the prevalence of these pathogens in turkey flocks in the future.

3. Materials and methods

3.1. Study Population

Two turkey flocks of the line British United Turkey (BUT-Big 6) were investigated, comprising 1,200 males and 4,500 female birds. Both were kept in the same house separated by physical barriers. The male flock was in the department close to the main entrance of the

house and the females were kept in the back department. The 1-d-old poults were obtained from the same hatchery. Rearing, feed, and management conditions were identical.

3.2. Sampling Schedule

Fecal samples (60 randomly selected dropping samples from each flock) were collected at several intervals (Table 1) according to Gregory et al. (1997), pooled in 6 tubes (each contained 10 samples), and transported to the laboratory for analysis within 30 min. Samples were investigated using mPCR parallel with isolation for all fecal, feed, and drinking water samples. Environmental samples (30 representative samples pooled in 6 tubes) were taken from different sections of the turkey house (Table 1). First, samples were taken 2 d before restocking and then regularly over the rearing period. Drinking water, feed, litter, fans, and worker boots were investigated. Walls, windows, and truck tires were swabbed with cotton swabs and investigated once at the beginning of the study.

Table 1. Detection of *Campylobacter* during turkey rearing using multiplex PCR[1]

	Female group						Male group					
Experimental day	Fecal sample pools	Drinking water	Feed	Litter	Fan	Worker boots	Fecal sample pools	Drinking water	Feed	Litter	Fan	Worker Boots
−2 d	ND[2]	0/6	ND	0/6	0/6	0/6	ND	0/6	ND	0/6	0/6	0/6
1 d	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
3 d	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
6 d	0/6	2/6	0/6	0/6	0/6	0/6	0/6	1/6	0/6	0/6	0/6	0/6
13 d	1/6	4/6	1/6	4/6	0/6	0/6	0/6	3/6	1/6	4/6	0/6	0/6
20 d	2/6	4/6	3/6	6/6	0/6	0/6	1/6	4/6	3/6	4/6	0/6	0/6
27 d	2/6	6/6	2/6	ND	ND	ND	2/6	6/6	3/6	ND	ND	ND
38 d	5/6	ND	ND	ND	ND	ND	4/6	ND	ND	ND	ND	ND
59 d	6/6	ND	ND	ND	ND	ND	4/6	ND	ND	ND	ND	ND
71 d	1/1[3]	ND	ND	ND	ND	ND	5/6	ND	ND	ND	ND	ND
85 d	1/1[3]	ND	ND	ND	ND	ND	1/1[3]	ND	ND	ND	ND	ND
106 d	1/1[3]	ND	ND	ND	ND	ND	1/1[3]	ND	ND	ND	ND	ND

Number of positive samples per total number of collected samples

[&]ND = no samples were investigated

[!] Each pool contained 10 samples

3.3. DNA Extraction

Genomic DNA was extracted directly from feces and environmental samples with the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 5 g of fecal matter were placed in a plastic bag (Oxoid, Germany), and then 5 mL of PBS were added and well-mixed in a stomacher (Seward stomacher 80, Biomaster Lab System, Worthing, UK) at normal speed for 1 min. Two-hundred microliters of the previous mixture were transferred into a 2.0-mL safe-lock tube (Eppendorf, Hamburg, Germany); 200 µL of tissue lysis buffer and 40 µL of proteinase K (Roche Diagnostics GmbH) were added and vortexed. Incubation for 60 min at 55°C followed. Further DNA extraction was carried out according to the manufacturer's instructions using the protocol for isolation of nucleic acids from mammalian tissue of the Roche commercial kit. The yielded DNA can be used directly or stored at −20°C for later analysis. The DNA extractions from the environmental samples were performed the same as for the fecal matter.

3.4. mPCR

A modified version of an mPCR assay (Denis et al., 1999) was used to identify members of the genus *Campylobacter* and of the 3 thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*). Primer sequences are given in Table 2.

Table 2. Multiplex PCR for the detection of members of the genus and of the thermophilic species of *Campylobacter*

Target gene	Primer (pmol/μL)	Sequence (5′-3′)	Amplicon length specificity	Reference
16S rRNA	MD16S1 (2.5) MD16S2 (2.5)	ATC TAA TGG CTT AAC CAT TAA AC GGA CGG TAA CTA GTT TAG TAT	857 bp *Campylobacter*	Denis et al., 1999
mapA	MDmapA1 (10) MDmapA2 (10)	CTA TTT TAT TTT TGA GTG CTT GTG GCT TTA TTT GCC ATT TGT TTT ATT A	589 bp *Campylobacter jejuni*	Denis et al., 1999
ceuE	MDCOL2 (10) MDCOL3 (10)	TGA TTT TAT TAT TTG TAG CAG CG AAT TGA AAA TTG CTC CAA CTA TG	462 bp *Campylobacter coli*	Denis et al., 1999
16S rRNA	CL 1155 R (10) CL 632-rv (10)	ATT TAG AGT GCT CAC CCG AAG GGG AAA CTG GTA ATC TAG AGT GG	522 bp *Campylobacter lari*	This study

The PCR was performed in a 50-μL reaction mixture containing 5.0 μL of 10× Taq reaction buffer complete (Jena Bioscience GmbH, Jena, Germany), 2.0 μL of dNTP mix (2 m*M* each; Carl Roth GmbH, Karlsruhe, Germany), 2.0 μL of each primer (Jena Bioscience GmbH; Table 2), and 0.2 μL of Taq Pol thermostable DNA polymerase (Jena Bioscience GmbH). Amplification reactions were carried out in a TRIO Thermoblock cycler (Biometra, Göttingen, Germany) using the following program: one cycle of 1 min at 96°C followed by 35 cycles each consisting of 60 s at 95°C, 90 s at 59°C, and 60 s at 72°C. The PCR was terminated by a final extension step of 5 min at 72°C. Amplification generated 857, 589, 522, and 462 base pair DNA fragments specific for the genus *Campylobacter* and the species *C. jejuni*, *C. lari*, and *C. coli*, respectively. For analysis, 20 μL of PCR products were subjected to electrophoresis in a 1.5% agarose gel for 1 h, stained with ethidium bromide (0.5 μg/mL), and visualized under UV light. Results were documented using BioImage system GeneGenius (Syngene, Synoptics Ltd., Cambridge, UK). Reference strains *C. jejuni* DSM 4688, *C. coli* DSM 4689, and *C. lari* DSM 11375 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were used as positive controls.

3.5. Isolation and Identification of *Campylobacter*

Isolation and identification of thermophilic *Campylobacter* from poult feces, drinking water, and feed samples were performed according to ISO 10272–1 (ISO, 2006). Modified CCDA plates (Oxoid, Wesel, Germany) and brilliance CampyCount agar (Oxoid Deutschland GmbH) were used for *Campylobacter* isolation under microaerophilic conditions (5% $O_{\&}$ 10% $CO_{\&}$ and 85% $N_{\&}$) in gas pack jars at 37°C for 48 h after enrichment in Bolton broth (Oxoid). Suspected colonies were identified phenotypically by Gram staining and the biochemical reaction profiles obtained by the API Campy System (bioMerieux Deutschland GmbH, Nürtingen, Germany) used according to the instructions of the manufacturer.

4. Results

Before restocking, the environment of the cleaned and disinfected turkey house was investigated for the presence of *Campylobacter* DNA using an mPCR assay. All samples obtained from swabbed surfaces of walls, windows, fans, truck tires, and other matrices, such

as drinking water and litter, were negative in these investigations (Table 1). The first positive PCR results were achieved at d 6. *Campylobacter* DNA was detected in drinking water of both groups. Bacteriological examinations, however, revealed negative results. At d 13, DNA of thermophilic *Campylobacter* was also found in feed and litter of both groups as well as in the fecal samples collected from female birds. The colonization of the males started later. By d 59, *Campylobacter* DNA could be detected in all fecal samples collected from female birds. This could be observed in the male group at d 85 (Table 1). In summary, birds became colonized between the second and the third week. By the fourth week of age, the environmental samples, including drinking water and feed, were positive for *Campylobacter*. No positive findings were obtained from workers' boots during the whole investigation time. *Campylobacter jejuni* and *C. coli* were identified in the fecal samples by mPCR (Figure 1). First findings were exclusively *C. jejuni*. Later on, detection of *C. coli* followed. Because of pooling the samples, exact data cannot be given for the presence of *C. jejuni* and *C. coli*. Mixed infections in single birds could not be excluded. *Campylobacter lari* was not detected. The detection limit for *C. jejuni*, *C. coli*, and *C. lari* using mPCR under described conditions was < 10 pg of DNA. Nineteen out of 51 investigated fecal pool samples from female birds revealed positive results (37.3%), whereas only 18 out of 61 fecal samples collected from males were positive (29.5%). Fourteen *C. jejuni* strains were isolated from fecal material during the investigation time.

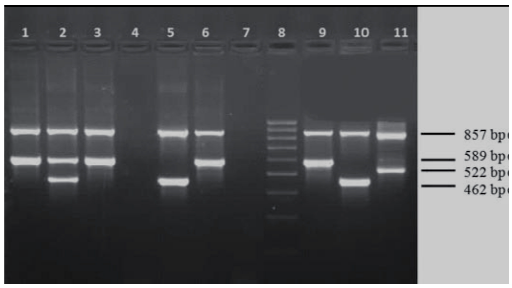

Figure 1. Results of investigation of fecal samples using multiplex PCR (female group; 38 d of age). Lane 1 = pool 1, *Campylobacter jejuni*; lane 2 = pool 2, mixed infection *C. jejuni* and *Campylobacter coli*; lane 3 = pool 3, *C. jejuni*; lane 4 = pool 4, negative result; lane 5 = pool 5, *C. coli*; lane 6 = pool 6, *C. jejuni*; lane 7 = negative control; lane 8 = 100-bp marker; lanes 9 to 11 = positive controls (*C. jejuni* DSM 4688, *C. coli* DSM 4689, and *Campylobacter lari* DSM 11375, respectively).

5. Discussion

Occurrence of thermophilic *Campylobacter* species in German turkey production is widespread and of human public health concern (Hafez et al., 2001; Alter et al., 2005). This study focused on the use of mPCR as a rapid molecular method for the estimation of the prevalence and detection of the start point of colonization in turkey flocks of different sexes. *Campylobacter* is extremely susceptible to multiple stresses and is considered to be quite vulnerable outside the host; moreover, survival in pure culture can be limited to minutes depending on the stress and environmental conditions (Obiri-Danso et al., 2001). Many other food-borne pathogens, including *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterobacter* have an ability to form a biofilm, an assemblage of bacteria encased in a sticky polymer, that provides protection from

desiccation, sanitizers, antibiotics, and multiple environmental stresses, whereas *Campylobacter* has been shown to be a poor biofilm initiator (Hanning et al., 2008).

As a result of the fastidiousness and diversity of campylobacters, it is clear that no one medium will provide an accurate measure of their occurrence. In the present study, information can be given only about *Campylobacter* DNA inside the farm originating either from dead or live bacteria. For this reason, the use of PCR-based detection methods is very attractive. The birds were obtained from the same breeding source and raised under the same environmental conditions. The numbers of fecal dropping samples (60) from each male and female turkey group were sufficient to find the organism at a ≥ 5% level of prevalence with a 95% level of confidence (Gregory et al., 1997). Also, the number of environmental samples (30) was sufficient to find the organism at a ≥ 10% level of prevalence and 95% confidence. Our results proved that all samples, either environmental or from birds, were negative for *Campylobacter* during the first week of breeding, demonstrating 2 facts: 1) the presence of strict biosecurity after cleaning and disinfection of the barn (Ruckaberle et al., 1999; Evans and Sayers, 2000; Petersen et al., 2001) and 2) that there is no vertical transmission or contamination with *Campylobacter* at the hatchery. These data were supported by other studies showing that birds are not colonized at the time of hatch and *Campylobacter* are rarely isolated from chicks younger than 14 d (Pokamunski et al., 1986; Gregory et al., 1997; Evans and Sayers, 2000; Hafez et al., 2001; Sahin et al., 2003; Wesley and Muraoka, 2011).

During daily analysis of drinking water and feed samples, the first detection of *Campylobacter* DNA was successful in water at d 6 after restocking. The source of the contamination remained unclear. It can be hypothesized that drinking water is the primary source of contamination, being in agreement with the results of Bull et al. (2006) and Sasaki et al. (2011). Infection of poultry by artificially infected drinking water is possible (Shanker et al., 1990). On the other hand, the hypothesis of water as a contamination source is challenged by the results of Pokamunski et al. (1986) and Patriarchi et al. (2009), who could not recover *Campylobacter* from any water sample of any farm they surveyed. In the present study, the drinking water was not treated with any disinfectants or antibiotics. The bell-type drinkers in the farm were supplied through pipes from an open water tank that was placed outside of the turkey house in a separate room. Therefore, the contamination of the drinking water source by insects is possible, but unfortunately we did not examine insects for the presence of *Campylobacter*. The possibility of biofilm formation cannot be excluded and it may be supposed to enhance the *Campylobacter* contamination.

Polymerase chain reaction inhibitors as heme degradation products, such as bilirubin, as well as bile salts, polysaccharides, and large amounts of irrelevant DNA (Lund et al., 2003) are substances that inhibit enzyme activity. They are present in many biological samples and can limit the advantages of PCR detection. Various strategies can be applied to circumvent the problem of inhibitors; for example, optimization of the DNA isolation procedure to exclude inhibitors prior to the amplification and use of *Taq* DNA polymerase with a high capacity for amplification in the presence of the actual inhibitors (Lund et al., 2003). We found that the inhibitory removal solution present in the commercial DNA isolation kit performed very well with fecal samples. Combined with amplification using a high-yield *Taq* DNA polymerase containing glycerol that neutralizes the effect of inhibitors (Nagai et al., 1998), the problem of inhibitors in fecal material was greatly reduced in PCR. The significance of sex regarding the

time of colonization with *Campylobacte*r has not been frequently addressed in the literature. In this study, it was noted that at the beginning of the second week of age, a positive result was obtained from cloacal swabs of the female group, followed by the first positive result in the male group a week later. In addition, a higher prevalence in the female group was seen than in the male group. In contrast, Cox et al. (2000) found no differences in the time of colonization of males and females and that the prevalence in both groups was similar. The role of sex in the context of susceptibility was discussed before. Differences were found between male and female turkeys in susceptibility to an *Escherichia coli* challenge and against physical stress (Huff et al., 1999, 2007). As food safety is an increasing demand from consumers, there is a need for simple, rapid, and cheap methods for the detection of zoonotic agents. We demonstrated that mPCR is able to fulfill these requirements for the investigation of feces and environmental samples in field trials when compared with conventional culture methods.

The present result could be obtained in < 330 min (90 min of DNA extraction, 180 min of mPCR amplification, and 60 min of electrophoresis). With the method presented here, the *Campylobacter* status of a flock can be revealed within the same day, in comparison with conventional isolation methods that require pre-enrichment of fecal samples in nutrient broth for 24 h, followed by inoculation onto a selective medium under microaerobic incubation at 37°C or 42°C for 48 h. A further 48 h is required for full phenotypic identification. Our results are in agreement with studies of Gonzalez et al. (1997), Linton et al. (1997), Lawson et al. (1998), Denis et al. (1999, 2001), Lund et al. (2003, 2004), Wang et al. (2002) and Perko-Mäkelä et al. (2009). Multiplex PCR used under the conditions described allowed the detection of <10 pg of the 3 thermophilic *Campylobacter* species, which corresponds to 103 genome equivalents. This amount is sufficient for a reliable detection of *Campylobacter* DNA in fecal samples. The present method claimed to be a cheap method for monitoring *Campylobacter* status in a poultry farm because it cost for one sample 2.25 € (1.50 € for DNA extraction, 0.50 € for mPCR amplification, and 0.25 € for electrophoresis).

Start point of colonization and prevalence of *Campylobacter* in turkey flocks depend on age and sex. Strict hygienic biosecurity could not prevent colonization of poults by thermophilic *Campylobacter* but decreases the load of intestinal contamination and delays the onset of colonization. Great attention must be given to drinking water as a presumable source of *Campylobacter* contamination of a flock.

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C H A P T E R 3

Genetic dynamics and diversity in *C. jejuni* in turkey flock

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Detection of Genetic Diversity in *Campylobacter jejuni* Isolated from a Commercial Turkey Flock Using *flaA* Typing, MLST Analysis and Microarray Assay

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1. Summary

Campylobacter is genetically highly diverse and undergoes frequent intraspecific recombination. Turkeys have been identified as an important reservoir for *Campylobacter jejuni* which is of public health significance. The assessment of the genetic diversity among *Campylobacter* population is critical for our understanding of the epidemiology of this bacterium. The genetic profiles were different according the used molecular typing methods. The performance of established *fla*A genotyping, multilocus sequencing typing (MLST) and DNA microarray assay based on the ArrayTube™ technology was evaluated using 14 *Campylobacter jejuni* isolated from a commercial turkey flock. The *fla*A typing was performed using PCR-RFLP with restriction enzymes *Sau3*AI, *Alu*I, a 'composite' *fla*A analysis of *Alu*I and *Sau3*AI and *Dde*I. The 14 isolates were differentiated into 3, 5, 7 and 9 genotypes, respectively. Entire *fla*A gene and short variable region (SVR) sequences were analysed. Sequencing of the entire *fla*A provided 11 different genotypes. *fla*A-SVR sequence analysis detected 8 *fla*A alleles and 4 *fla*A peptides. One new *fla*A allele type (528) was identified. MLST analysis represented 10 different sequence types (STs) and 6 clonal complexes (CCs). The microarray assay recognised 14 different genotypes. The discriminatory indices were 0.560, 0.802, 0.857, and 0.912 for *fla*A-RFLP depending on the used enzymes, 0.890 for *fla*A-SVR, 0.967 for entire *flaA* sequencing, 0.945 for MLST and 1.00 for the DNA microarray assay. The *fla*A gene was genetically stable over 20 passages on blood agar.

In conclusion, the different typing tools demonstrated a high level of genetic heterogeneity of *Campylobacter jejuni* in a turkey flock indicating that a single flock can be infected by multiple genotypes within one rearing cycle. DNA microarray-based assays had the highest discriminatory power when compared with other genotyping tools.

2. Introduction

Campylobacter is recognized as the leading cause of bacterial gastroenteritis in Europe and a significant public health concern worldwide. Poultry and poultry products remain the most important source of food-borne human campylobacteriosis (EFSA, 2011; Gu et al., 2009). Genetic diversity among thermophilic *Campylobacter* spp. may enable survival of these bacteria in the environment by means of variation in strain virulence (Parkhill et al., 2000). Advanced molecular typing tools have improved our understanding of the epidemiology of

bacterial food-borne pathogens. Monitoring of poultry flocks has shown that some are infected with only one genotype of *Campylobacter* spp., while more than one genotype has been detected in others (Alter et al., 2011; Hafez et al., 2001). Different strains in individual flocks may be replaced or displaced by others during the rearing cycle (Petersen and Newell, 2001; Thomas et al., 1997). Some clones of *C. jejuni* remain genetically stable in completely different environments over long periods of time and considerable geographical distances. Moreover, the human isolates remained stable for almost 20 years under laboratory conditions (Manning et al., 2001).

There are various methods used for typing *C. jejuni* (Wassenaar and Newell, 2000). It has been suggested that the sensitivity of the *flaA* gene locus to spontaneous genetic change is a limiting factor in its use in long-term epidemiological studies, but is suitable for the initial grouping of isolates in surveillance situations (Petersen and On, 2000). The *flaA* gene of *Campylobacter* species serves as an epidemiological marker, as it shows extensive sequence heterogeneity (Wassenaar and Newell, 2000). The *flaA* typing in *C. jejuni* is a commonly used, rapid and easy method for genotyping with an acceptable discriminatory power (Petersen and Newell, 2001; Behringer et al., 2011; Meinersmann et al., 1997). It has been shown that PCR-RFLP of *flaA* amplicons was suitable for discriminating *C. jejuni* isolates by generating DNA banding pattern (Nachamkin et al., 1993). Different restriction enzymes can be used, and combining the enzyme patterns (composite analysis) has been shown to result in an increased degree of discrimination (Wassenaar and Newell, 2000; Harrington et al., 2003). Sequencing of the entire *flaA* gene is a highly reproducible method, allowing precise and simple worldwide comparison of isolates (Colles et al., 2003). The entire coding sequence of *flaA* gene (1,764 nucleotides) of *C. jejuni* contains two regions of high variability, one region from approximately base positions 700 to 1,450 and a short variable region (SVR) from base positions 450 to 600 (Meinersmann et al., 1997; Foley et al., 2009).

Multilocus sequence typing (MLST) is suitable for the investigation of diverse bacterial populations which have weakly clonal population structures (Suerbaum et al., 2001; Dingle et al., 2001). The MLST scheme displays high portability and great facility for inter-laboratory comparisons, which has contributed to a greater understanding of the population structure and global epidemiology of *C. jejuni* and related organisms (Clark et al., 2012).

C. jejuni has approximately 1300 core and house-keeping genes that encode functions required for survival, as determined by comparative genomic sequencing (Fouts et al., 2005) and by microarray-based comparative genomic hybridization analysis (Pearson et al., 2003; Taboada et al., 2004). Whole genome DNA microarrays are used to investigate the genomic dynamics through determination the presence or absence of thousands of genes in a single hybridization experiment. They are suitable for rapid and accurate simultaneous differentiation among thermophilic campylobacters (Taboada et al., 2007; Volokhov et al., 2003). The ArrayTube™ (AT^{TM}) system is a less expensive platform and characterizes *C. jejuni* isolates by specific hybridization patterns of selected gene loci. The advantage of the AT^{TM} system is enzyme-catalysed precipitation staining rather than fluorescence detection of positive hybridization signals; moreover, the signal intensities are measured by a simple transmission technique (Buchanan et al., 2011; Ehricht et al., 2006).

The discriminatory power of the different genotyping methods was determined by the measure of resolution that could be achieved by the respective methods. It is given as a

numerical value, which can be used for simple comparison between methods (Hunter and Gastom, 1988).

The objective of this study was to determine the genetic diversity among *C. jejuni* isolates recovered from a single turkey flock during the production cycle. Sampling and cultivation of *C. jejuni* isolates were carried out from the beginning of flock colonization until slaughter. The *C. jejuni* heterogeneity was investigated using different genotyping tools such as *flaA* typing, MLST and microarray analysis. The performance of the different genotyping methods was evaluated based on their discriminatory power, costs per isolate, ease of handling, and time-to-result.

3. Materials and methods

3.1. Bacterial strains and growth conditions

Fourteen *C. jejuni* isolated from cecal content of a turkey flock reared in a single farm. The flock was sampled at several dates (Table 1) from the beginning until the end of the production cycle. The isolation was carried out according to ISO 10272 (ISO, 2006). Briefly, 1 g of fecal sample was inoculated into 9 ml of Bolton selective enrichment broth (Oxoid Deutschland GmbH, Wesel, Germany) and incubated at 42ºC for 24 h under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) (Jenny medical-Trilab, Schütt Labortechnik, Göttingen, Germany). A loop of broth was plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid Deutschland GmbH) and Brilliance CampyCount Agar (Oxoid Deutschland GmbH). Incubation was done at 37ºC for 6 h followed by incubation at 42ºC for 18 - 36 h under microaerophilic conditions. Suspected colonies were subcultured on Mueller Hinton (MH) blood agar (10% citrated bovine blood). Bacterial cultures were identified phenotypically by Gram staining and by the API 20E system (bioMerieux Deutschland GmbH, Nürtingen, Germany).

Table 1. Restriction profiles of *flaA* typing, *flaA*-SVR alleles (321 nucleotides) and *flaA*-SVR peptides (107 peptides) of 14 *C. jejuni* isolates, their accession numbers and the date of isolation

Isolates	*Sau3AI* (A–C)	*AluI* (1–5)	*AluI*/*Sau3AI* (I–VII)	*DdeI* (a–i)	*flaA*-SVR alleles	*flaA*-SVR peptides	Accession No.	Date of isolation
CS0048	B	4	I	a	105	1	JQ991581	04-05-10
CS0052	A	1	II	b	515	1	JQ991582	17-06-10
CS0073	A	4	IV	c	18	20	JQ991583	07-07-10
CS0074	A	3	III	d	1124	1	JQ991584	07-07-10
CS0075	B	4	V	e	34	1	JQ991585	07-07-10
CS0076	A	3	III	d	1124	1	JQ991586	07-07-10
CS0077	A	3	III	d	1124	1	JQ991587	07-07-10
CS0078	C	2	VI	f	359	9	JQ991588	07-07-10
CS0079	B	4	V	g	34	1	JQ991589	07-07-10
CS0080	A	3	III	d	1124	1	JQ991590	07-07-10
CS0081	A	1	II	b	515	1	JQ991591	07-07-10
CS0082	A	1	II	h	515	1	JQ991592	21-07-10
CS0083	A	1	II	h	528	1	JQ991593	21-07-10
CS0084	C	5	VII	i	16	12	JQ991594	21-07-10

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3.2. DNA Extraction

Genomic DNA was extracted from a 48 h bacterial culture on MH blood agar plates using High Pure PCR Template Preparation Kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The DNA was eluted in 200 µl elution buffer. DNA was quantified spectrophotometrically using a Nanodrop[1] ND-1000 (Fisher Scientific GmbH, Schwerte, Germany).

3.3. Species confirmation and *fla*A-RFLP assays

The identified isolates were confirmed as *C. jejuni* by using a multiplex PCR (mPCR) assay as described previously (El-Adawy et al., 2012). For *fla*A-RFLP analysis extracted DNA was amplified, as described elsewhere (Nachamkin et al., 1993), using modified primers with nucleotide sequences as given in Table 2. Amplification conditions were: initial denaturation for 60 s at 94°C followed by 35 cycles each consisting of 15 s at 94°C, 60 s at 45°C, 120 s at 72°C and a final extension step of 300 s at 72°C. The *fla*A amplicon was digested for 18 h at !. ^{o}C with *Alu*I (Jena Bioscience GmbH, Jena, Germany), *Dde*I (Roche Diagnostics GmbH), *Sau3*AI (Jena Bioscience GmbH), and a mixture of *Sau3*AI and *Alu*I enzymes using the incubation buffer recommended by the manufacturers. The DNA segments were separated using 2.5% agarose gels (Starlab GmbH, Hamburg, Germany) in TBE buffer at 200 V for 1 h, stained with ethidium bromide and visualized under UV light. Documentation was done using a Bio Imaging System (Syngene, Cambridge, UK).

3.4. Analysis of *fla*A-RFLP results

TIF images of the restriction profiles for *flaA*-RFLP were incorporated for analysis into BioNumerics V. 4.50 (Applied Maths, Austin, TX, USA). Pair comparisons and cluster analysis were made using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. The optimization and position tolerance for band analysis were set at 4%, and a cut-off of 90% was used for the determination of the different restriction patterns for *flaA*-RFLP.

3.5. *In vitro* stress model (genetic stability test)

Seven *C. jejuni* isolates derived from single colonies (CS0048, CS0052, CS0073, CS0077, CS0078, CS0079, and CS0084) with different restriction profiles of the *flaA* genes digested with *Dde*I were selected for stress test. Isolates were stored in cryovials (Mast Diagnostica Laboratoriums-Präparate GmbH, Reinfeld, Germany) at -80ºC after the first isolation, and none were subcultured or cloned prior to analysis. The isolates were grown on MH blood agar plates (Oxoid Deutschland GmbH) supplemented with 10% citrated bovine blood and incubated for 24 h at 42°C under microaerophilic conditions (5% $O_{\&}$ 10% $CO_{\&}$ 85% $N_{\&}$) (Jenny medical-Trilab, Schütt Labortechnik). Isolates were then subcultured 20 times on MH blood agar for 48 h at 42°C in a microaerophilic atmosphere. After the 4th, 8th, 12th, 16th and &:th passage, chromosomal DNA was extracted from selected isolates of these passages and subtyped by *fla*A-RFLP as described above.

3.6. DNA sequencing of the entire *fla*A gene

The *fla*A amplicons of all isolates with a length of approximately 1.7 kb were excised from the gel and DNA was purified using the QIAamp Gel Extraction Kit (Qiagen, Hilden,

Germany) according to the manufacturer´s recommendations. Cycle sequencing was done with different sequencing primers (Table 2) using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the recommendations of the manufacturer. Sequencing products were analyzed with a Genetic Analyzer ABI PRISM 3130 (Applied Biosystems). Whole *fla*A gene and short variable region (SVR) sequences were analyzed to identify the most parsimonious relationships. Based upon sequence data (accession numbers in Table 1) of investigated isolates a split network tree was constructed with cluster tree neighbour-joining analysis using the bioinformatics tools of Geneious V5.1 analysis (Drummond et al., 2010). Dendrograms were generated for both the entire *flaA* gene sequence and the *fla*A-SVR sequence (bases 450 through 600). This approach is compatible with the sequence typing and schemes used in the PubMLST database (http://www.pubmlst.org/). The *flaA*-SVR alleles (321 nucleotides from position 280 to 600) and peptides (n = 107) were calculated using the database available at (http://pubmlst.org/campylobacter).

Table 2. Primers used for *flaA* typing and MLST of *C. jejuni* isolates

Primer	Sequence	Gene	Aim	Amplicon bp
flaA1-Wob	5′-GGATTTCGTATTAACA-3′	*flaA*	Amplification	~1,700
fla 2-Wob	5′-CTGTARYAATCTTAAAACATTTTG-3′	*flaA*	Amplification	~1,700
flaA-S-1	5′-GCAGATGATGCTTCAGGG-3′	*flaA*	Sequencing	
flaA-S-2	5′-CTGCTATCGCCATCCCTG-3′	*flaA*	Sequencing	
flaA-S-3	5′-AAATCAAGTYACATCRAC-3′	*flaA*	Sequencing	
flaA-S-4	5′-AGAGTARTTTGCACTCTC-3′	*flaA*	Sequencing	
flaA-S-5	5′-GATAAGGCTATGGATGAGC-3′	*flaA*	Sequencing	
flaA-S-6	5′-GCTCTGATTTGATCAAG-3′	*flaA*	Sequencing	
flaA-S-8	5′-AAGTGTGGTAACACCTGC-3′	*flaA*	Sequencing	
flaA-S-9	5′-CCYACWGAAWAWCCYGAACC-3′	*flaA*	Sequencing	
flaA-S-10	5′-TCAAGAATTTCAAATCGG-3′	*flaA*	Sequencing	
flaA-S-11	5′-AAAKCCCATAGCATCRGC-3′	*flaA*	Sequencing	
flaA-S-12	5′-TTACTCTTAAAAACTAC-3′	*flaA*	Sequencing	
flaA-S-13	5′-CCATCATTTTTAACTAAA-3′	*flaA*	Sequencing	
asp-A9	5′-AGTACTAATGATGCTTATCC-3′	*aspA*	Amplification	899
asp-A10	5′-ATTTCATCAATTTGTTCTTTGC-3′	*aspA*	Amplification	899
asp-S3	5′-CCAACTGCAAGATGCTGTACC-3′	*aspA*	Sequencing	
asp-S6	5′-TTAATTTGCGGTAATACCATC-3′	*aspA*	Sequencing	
gln-A1	5′-TAGGAACTTGGCATCATATTACC-3′	*glnA*	Amplification	1,262
gln-A2	5′-TTGGACGAGCTTCTACTGGC-3′	*glnA*	Amplification	1,262
gln-S3	5′-CATGCAATCAATGAAGAAAC-3′	*glnA*	Sequencing	
gln-S6	5′-TTCCATAAGCTCATATGAAC-3′	*glnA*	Sequencing	
glt-A1	5′-GGGCTTGACTTCTACAGCTACTTG-3′	*gltA*	Amplification	1,012
glt-A2	5′-CCAAATAAAGTTGTCTTGGACGG-3′	*gltA*	Amplification	1,012
glt-S1	5′-GTGGCTATCCTATAGAGTGGC-3′	*gltA*	Sequencing	
glt-S6	5′-CCAAAGCGCACCAATACCTG-3′	*gltA*	Sequencing	
gly-A1	5′-GAGTTAGAGCGTCAATGTGAAGG-3′	*glyA*	Amplification	816
gly-A2	5′-AAACCTCTGGCAGTAAGGGC-3′	*glyA*	Amplification	816
gly-S3	5′-AGCTAATCAAGGTGTTTATGCGG-3′	*glyA*	Sequencing	
gly-S4	5′-AGGTGATTATCCGTTCCATCGC-3′	*glyA*	Sequencing	
pgm-A7	5′-TACTAATAATATCTTAGTAGG-3′	*pgm*	Amplification	1,150
pgm-A8	5′-CACAACATTTTTCATTTCTTTTTC-3′	*pgm*	Amplification	1,150
pgm-S2	5′-TCCAGAATAGCGAAATAAGG-3′	*pgm*	Sequencing	
pgm-S5	5′-GGTTTTAGATGTGGCTCATG-3′	*pgm*	Sequencing	
tkt-A3	5′-GCAAACTCAGGACACCCAGG-3′	*tkt*	Amplification	1,102
tkt-A6	5′-AAAGCATTGTTAATGGCTGC-3′	*tkt*	Amplification	1,102
tkt-S4	5′-ACTTCTTCACCCAAAGGTGCG-3′	*tkt*	Sequencing	
tkt-S5	5′-GCTTAGCAGATATTTTAAGTG-3′	*tkt*	Sequencing	
unc-A7	5′-ATGGACTTAAGAATATTATGGC-3′	*uncA*	Amplification	1,120
unc-A2	5′-GCTAAGCGGAGAATAAGGTGG-3′	*uncA*	Amplification	1,120
unc-S4	5′-TGCCTCATCTAAATCACTAGC-3′	*uncA*	Sequencing	
unc-S5	5′-TGTTGCAATTGGTCAAAAGC-3′	*uncA*	Sequencing	

primers created for this study.
primers according to [18].
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3.7. Multilocus sequence typing (MLST)

The MLST protocol was carried out as described previousl (Dingle et al., 2001). The target fragments of the housekeeping genes were *asp*A (aspartase), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxyl methyl transferase), *pgm* (phosphor glucomutase), *tkt* (transketolase), and *unc*A (ATP synthase alpha subunit). PCR products were amplified with designed oligonucleotide primer pairs (Table 2). The reaction conditions were: denaturation at 94°C for 120 s, primer annealing at 50°C for 60 s, and extension at 72°C for 60 s for 35 cycles. Amplicons were examined via gel electrophoresis and purified using the QIAamp Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer´s recommendations. Cycle sequencing and analysis of sequencing products were done as described above. Alleles, sequence types (STs) and clonal complexes (CCs) were assigned and putative phylogenetic relationships among the STs were presented using a minimum spanning tree, which was constructed using the MLST database available at (http://pubmlst.org/campylobacter).

3.8. Microarray DNA hybridization assay

The microarray typing was carried out based on gene loci which are absent or present in *C. jejuni* isolates using the ArrayTube^{TM} platform (Alere Technologies GmbH, Jena, Germany). Two types of AT^{TM} microarrays with spotted probes were used to differentiate *C. jejuni* isolates: *C. jejuni*-1 and Campy-2. The basic AT^{TM} sample processing and data analysis workflow were done using special commercial kits (Alere Technologies GmbH) according to the manufacturer´s instructions (www.alere-technologies.com). Briefly, 1 µg of RNA free genomic DNA was amplified by a duplex PCR using random primers and biotin-16-dUTP to label the amplicons. The amplified labeled DNA was hybridized to both arrays for one hour under agitation at 45°C, washed, and quantified after a colorimetric reaction using horseradish peroxidase and TrueBlue substrate.

3.9. Algorithm for the interpretation of array data

Hybridization signals were measured after 5 min precipitation using an ArrayTube transmission reader ATR-03 (Alere Technologies GmbH). Signals were processed using the Iconoclust software, version 2.3 in combination with the Partisan Array LIMS system which provided the specific algorithms. The photograph and raw signal intensity data were transferred to the Array Tube Analyzer software. Normalised intensities of the spots were calculated. The local backgrounds as well as spot intensities were measured, using only valid pixels within the automatically recognized spot area for the latter. Normalized intensities of spot values were calculated according to the following equation:

$$\text{Normalized intensity} = 1 - \frac{\text{Average intensity of all valid pixels of the spot}}{\text{Intensity of all valid pixels of the local background}}$$

Numerical data were translated into logical data using cut-off values. Resulting values below 0.1 were considered negative (-) and those above 0.3 were considered positive (+), values between 0.1and 0.3 were regarded as ambiguous (+/-). For further analysis, the average of all probes for a given target allele was used. Cut-off values were defined based on the average normalized intensities of biotin staining controls and the hybridization controls (Manning et al., 2001). The clustering of isolates was done based on the binary microarray data using the simple matching distance metric and UPGMA method of clustering in

http://genomes.urv.cat/UPGMA/index.php?entrada using 100% fingerprint similarity for cluster definition. To estimate the strain relationships at a slightly lower level of discrimination, data were also analyzed at the 90% fingerprint similarity level, using DendroUPGMA, a dendrogram construction utility which creates a dendrogram without recalculation from a set of variables organizing all types related at ≥ 90% into single clusters (Clark et al., 2012).

3.10. Evaluation of typing methods

Using the online tool for quantitative assessment of classification agreement (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool), the discriminatory power can be expressed by a numerical index of discrimination (D) as shown previously (Hunter and Gaston, 1988). D value of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, an index of 0.0 would indicate that all members of a strain population were of an identical type.

4. Results

All fourteen isolates were confirmed as *C. jejuni* by Gram staining, biochemical tests, and multiplex PCR. The *fla*A gene was amplified using a modified PCR primer system (Table 2). It resulted in approximately 1.7 kb amplicons for all isolates. The PCR-RFLP analysis of *flaA* genes of the 14 *C. jejuni* isolates revealed 3, 5, 7 and 9 genotypes when digested with *Sau3*AI, *Alu*I, and a combination of *Sau3*AI and *Alu*I, and *Dde*I, respectively (Table 1 and Figure 1).

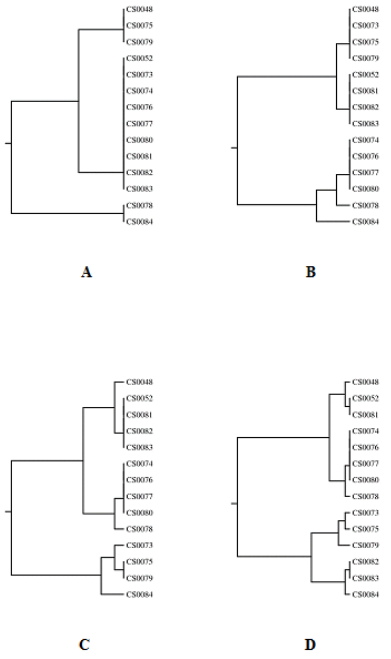

Figure 1. Dendograms based on restriction profiles of *flaA* genes. 14 *C. jejuni* isolates were digested using *Sau3*AI (A), *Alu*I (B), a combination of *Sau3*AI and *Alu*I (C), and *Dde*I (D). *fla*A-RFLP cluster analysis was performed with the Dice correlation coefficient and the unweighted pair group mathematical average clustering algorithm of BioNumerics ver. 4.50.

All isolates gave identical results when experiments were repeated (data not shown). *In vitro* stress tests demonstrated genetic stability of *flaA* genes in seven selected isolates over 20 subcultivations (Figure 2).

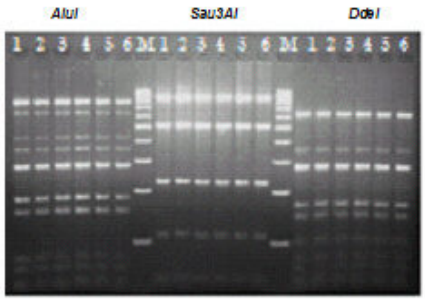

Figure 2. Agarose gel electrophoresis of PCR-RFLP profiles of *flaA* genes of *C. jejuni* isolate CS0078. Genetic stability was tested using *Alu*I, *Sau3*AI and *Dde*I. Lane M: 100 bp ladder (Jena Bioscience GmbH), lane 1: *fla*A-RFLP patterns before *in vitro* passage, lane 2 to lane 6: *fla*A-RFLP patterns after 4th, 8th, 12th, 16th and 20th passages, respectively.

DNA sequences of entire *fla*A genes of these *C. jejuni* isolates (sequences are available in GenBank with the accession numbers given in Table 1) were grouped into a single alignment and were analyzed for the most parsimonious relationships. The generated phylogenetic tree (Figure 3) had 11 terminal taxa, thereby each taxon was assigned by a numerical designation representing a unique sequence. The *fla*A sequences are characterized by a higher level of variability between sequence positions 700 and 1450. The results of *fla*A-SVR sequence typing of the 14 *C. jejuni* isolates are given in Table 1. Eight *flaA* alleles and 4 *fla*A peptides were detected. One new *fla*A allele type (528) was identified. The *fla*A-SVR dendrogram demonstrates a higher homology within these isolates than that obtained for the entire *fla*A genes (Figure 3).

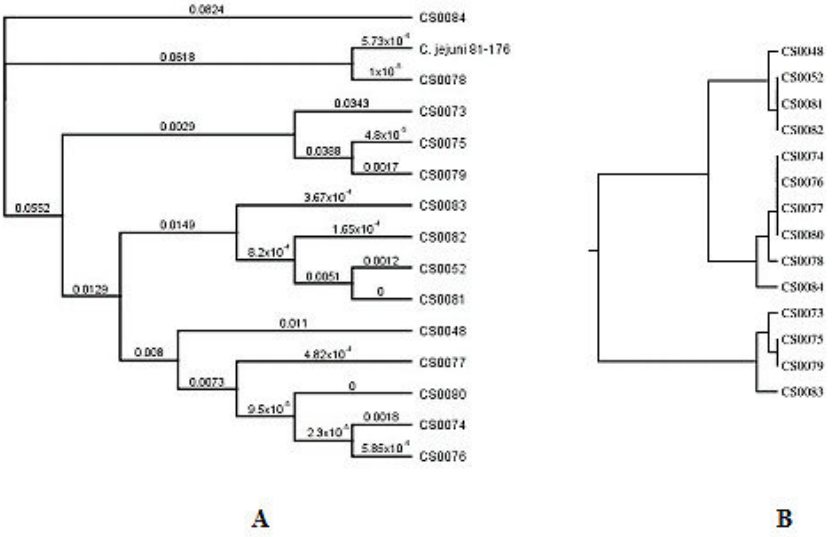

Figure 3. Relationships within 14 *C. jejuni* isolates based on entire *flaA* sequences (A) and *fla*A-SVR sequences (B). Dendrograms were generated using CLUSTREE neighbour-joining analysis. Scale bar: 0.02 divergent residues per site. Congruent topologies (P<25%) obtained using Geneious V5.1 (Drummond et al., 2010)

MLST analysis identified 10 STs, 7 of which (50, 5402, 604, 8, 905, 1409, 257) were present only once (Table 3).

Table 3. Allelic profiles and resulting sequence types (STs) and clonal complexes (CCs) for 14 *C. jejuni* isolates by using MLST analysis

Isolate	Allelic profile[A]							ST	CC
	aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
CS0048	2	1	12	3	2	1	5	50	ST-21
CS0052	2	115	298	26	127	29	35	4419	not found
CS0073	8	10	2	2	2	2	6	5402	ST-354
CS0074	2	1	1	3	2	1	5	21	ST-21
CS0075	47	55	5	10	11	48	8	450	ST-446
CS0076	2	1	1	3	2	1	5	21	ST-21
CS0077	2	1	1	3	2	1	5	21	ST-21
CS0078	1	2	3	27	5	9	3	604	ST-42
CS0079	47	55	5	10	11	48	8	450	ST-446
CS0080	2	1	1	3	2	1	6	8	ST-21
CS0081	2	115	298	26	417	29	35	4419	not found
CS0082	2	15	4	3	154	25	35	905	not found
CS0083	2	15	4	3	154	51	35	1409	not found
CS0084	9	2	4	62	4	5	6	257	ST-257

[A]http://pubmlst.org/campylobacter.
doi:10.1371/journal.pone.0051582.t003

The minimum spanning tree was constructed to show the relatedness among the 10 STs (Figure 4).

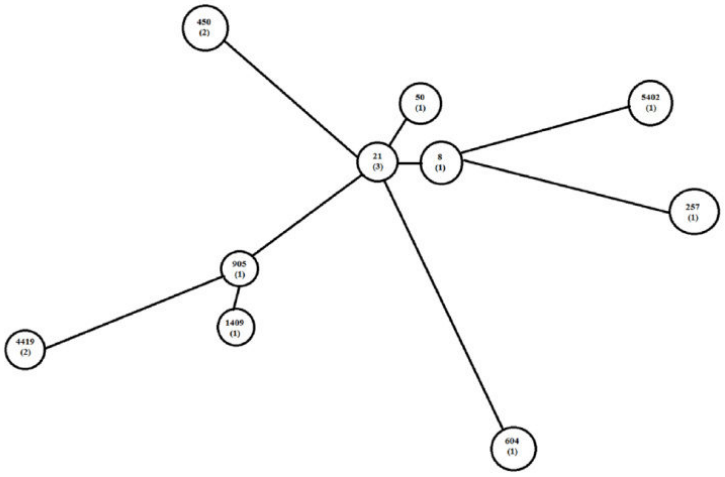

Figure 4. Minimum spanning tree depicting the clustering of 10 STs identified among 14 *C. jejuni* isolates. The tree was created using BioNumerics (version 4.6; Applied Maths). Each ST is represented by a circle. Numbers in brackets expressed numbers of isolates within a ST. The ST designations were obtained from http://pubmlst.org/campylobacter.

The dynamics of the colonization of the turkey flock by different *C. jejuni* isolates during the rearing process could be demonstrated (Table 2). The first *C. jejuni* ST 50 represented by CS0048 was substituted with other genotypes over a period of 11 weeks and could never be re-isolated. On the other hand, ST 4419 (CS0052) was isolated again after 5 weeks of the first finding (CS0081). At date 07.07.2010, 9 isolates were recovered which represented 5 coexisting STs. No dominating ST was found alongside the production process.

The microarray analysis showed a high level of discrimination (1.00) between *C. jejuni* isolates based upon different gene targets as shown in Figure 5.

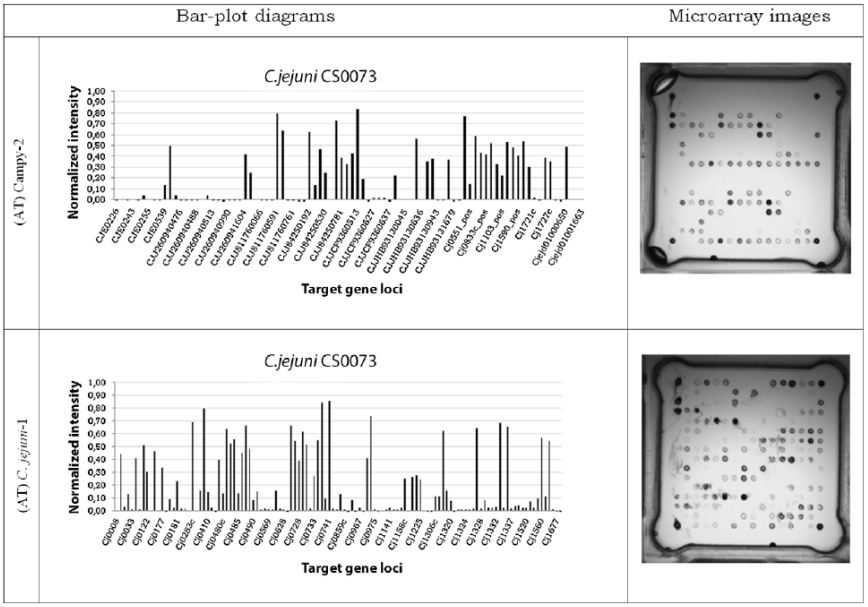

Figure 5. Hybridization patterns for *C. jejuni* isolate CS0073 presented as microarray images and bar-plot diagrams. The tested gene loci were arranged on two chips: *C. jejuni*-1 and Campy-2. The normalized intensity signal > 0.3 considered positive.

The microarray demonstrated as images and bar plot diagrams. The signal intensities express the absence or presence of different gene loci in the genome of *C. jejuni* isolates. The analysis of the hybridization images using the simple matching distance metric and UPGMA resulted in 14 different clusters. The dendrogram (Figure 6) illustrates the relatedness of the isolates based upon hybridization data.

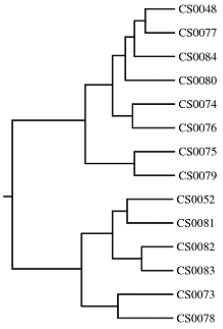

Figure 6. Dendrogram based on microarray data using DendroUPGMA. The clustering of *C. jejuni* isolates represents 14 different clusters based on the binary microarray data using the simple matching distance metric and (UPGMA) using average linkages.

The performance of the different genotyping systems was assessed based upon the index of discrimination, the costs per isolate, ease of handling, and time-to-result for one sample as shown in Table 4. Briefly, the highest discriminatory index was achieved using the microarray method (1.00). The entire *flaA* sequence analysis represented a higher discrimination (0.967) than MLST analysis (0.945) and *flaA*-SVR sequence analysis (0.890). The calculated D indices for the PCR-RFLP of the *flaA* genes with *DdeI* were higher (0.9121) than those for *Sau3AI, AluI* and the composite of *Sau3AI* and *AluI* digestion (0.5604, 0.8022, and 0.8571), respectively. The presented results could be obtained in 5 hours for the microarray assay, 10 hours for DNA sequencing, more than 18 hours for PCR-RFLP and 12 hours for the MLST assay. The PCR-RFLP method was found to be a cheap method for typing *Campylobacter* with a cost of 3.00 € per isolate, while the corresponding costs for entire *flaA* gene

sequencing, MLST and the microarray analysis reached 24.00 €, 70.00 € and 30.00 €, respectively.

Table 4. Comparison of the performance of *flaA* typing, MLST analysis and DNA microarray assay (ArrayTube™ technology) of 14 *C. jejuni* isolates

Typing technique	No. of types	Index of discrimination D [A]	CI (95%) [A]	CINA (95%) [A]	Time required (h)	Costs per sample (€)	Equipment
PCR-RFLP of *flaA*							PCR thermocycler
(a) *Sau3*AI	3	0.560	(0.325-0.796)	(0.311-0.810)			Electrophoresis
(b) *Alu*I	5	0.802	(0.722-0.882)	(0.683-0.922)	18	3.00	Incubator
(c) *Sau3*AI and *Alu*I	7	0.857	(0.754-0.961)	(0.727-0.988)			
(d) *Dde*I	9	0.912	(0.817-1.000)	(0.794-1.000)			
Entire *fla*A sequencing	11	0.967	(0.929-1.000)	(0.894-1.000)	10	24.00	PCR thermocycler Electrophoresis
*fla*A-SVR sequencing	8	0.890	(0.796-0.985)	(0.770-1.000)	10	15.00	Genetic analyzer
MLST analysis	10	0.945	(0.884-1.000)	(0.855-1.000)	12	70.00	PCR thermocycler Electrophoresis Genetic analyzer
DNA microarray assay	14	1.000	(1.000-1.000)	(0.946-1.000)	5	30.00	PCR thermocycler Thermomixer ArrayTubes (AT™) ArrayTube Reader

[A] The online tool at the Comparing Partitions website (http://www.comparingpartitions.info/) was used for this analysis. CI (95% confidence interval); CINA (95% non-approximated confidence interval)

5. Discussion

Thermophilic *Campylobacter* continues to significantly contribute to the worldwide public health impact. Understanding the epidemiology of *Campylobacter* spp. can help to reduce the disease burden. The genetic diversity amongst *Campylobacter* must be considered in epidemiological evaluations and microbial risk assessments of *Campylobacter* in poultry. Multiple genotypes can constitute the *Campylobacter* population within poultry flocks, suggesting different sources of exposure and/or genetic drifts within the *Campylobacter* population (Alter et al., 2011).

This study aimed to elucidate the genetic diversity among 14 *C. jejuni* isolates recovered from a single commercially reared turkey flock. These isolates were sampled from the beginning of the colonization until the slaughtering of the turkeys. Molecular biological typing was done using established *flaA* typing methods, MLST, and DNA microarray assay based on the ArrayTube™ technology. The usefulness of the different typing systems was evaluated.

The present study demonstrated that a single turkey flock can be simultaneously colonized with more than one *Campylobacter* genotype during the rearing cycle. It also reflected the changing in the occurrence of different types of *C. jejuni* between age 4 to 15 weeks. Types which were originally observed were substituted by others and could not be re-isolated anymore. Specific genotypes were also repeatedly identified at different rearing moments. Furthermore, investigations showed the simultaneous coexistence of different types at a single date. The dominant *C. jejuni* type could not be found in this turkey flock. No genotype was found which was present over the whole investigation period.

The situation in chickens was similarly described. Broiler flocks were identified in which different *Campylobacter* clones coexisted (Alter et al., 2011). In contrast, other studies (Ring et al., 2005; Chuma et al., 1997) reported the detection of only one genotype per sampled flock.

Molecular methods used for typing of *C. jejuni,* which are characterized by low complexity and high reproducibility, are needed to study the bacterial population structure.

The use of *fla*A gene typing for epidemiological studies is controversial, due to the intra- and inter-genomic recombination within the flagellin genes that results in significant sequence heterogeneity (Eberle and Kiess, 2012). PCR primers previously used for *fla*A amplification were found to be insufficient to amplify this gene in certain isolates (Petersen and Newell, 2001). Here, "wobbled" primers were used to amplify *flaA* genes. The discriminatory power of *flaA*-RFLP typing clearly depended upon the type of restriction enzyme used (Wassenaar and Newell, 2000; Eberle and Kiess, 2012). The results showed that cluster analysis based on composite digestion (*Alu*I and *Sau3*AI) of *fla*A genes was more discriminative than either single (*Alu*I or *Sau3*AI) enzyme *fla*A typing. Moreover, the highest discriminatory power in *fla*A-RFLP typing was achieved using *Dde*I. It was confirmed that *fla*A-RFLP typing should not be used alone to genotype the isolates (Behringer et al., 2011), due to the dependence of the results on the enzyme used and the limitation of the *flaA* gene being a very small part of the whole genome. PCR-RFLP assays are difficult to standardize and inter-laboratory comparisons of results are often ambiguous. DNA sequencing of entire *flaA* genes resulted in greater discriminatory power (D = 0.967) than that obtained with PCR-RFLP methods. DNA sequencing is done routinely in many research laboratories or is available as a low cost commercial service and the results can easily be exchanged among laboratories (Meinersmann et al., 1997; Nachamkin et al., 1993; Colles et al., 2003). In contrast to a previous report (Meinersmann et al., 1997), this study demonstrated that the genetic relatedness derived from the *fla*A-SVR sequence did not correlate with that obtained by the entire *fla*A gene sequence. The D index of *fla*A-SVR analysis was lower (D = 0.890) than that obtained by entire *fla*A gene sequencing (D = 0.967).

MLST is an important technique that provides a reliable prediction of clonality for population studies of *Campylobacter* spp. with high discrimination Dingle et al., 2001; Clark et al., 2012; Eberle and Kiess, 2012). Furthermore, another benefit of MLST is that assignment of DNA sequences to MLST alleles and sequence types is not prone to the variation and interpretation of restriction band profiling and band migration through the electrophoretic medium and MLST sequence data can be readily compared between laboratories (Clark et al., 2012). In this study, the MLST analysis represented 10 different sequence types and 5 clonal complexes. Two identified STs (ST 450 and ST 257) were previously reported in turkey. Other STs (50, 4419, 21, 604, 905 and 1409) were observed from chicken and from human cases of illness; ST 5402 was detected in pork offal and ST 8 was detected in cattle and sheep (http://www.pubmlst.org/campylobacter). Although MLST results are easy to reproduce, interpret and transfer, it is a complex, labor-intensive and expensive technique to perform in comparison with other typing methods used in this study as well as described earlier (Lévesque et al., 2008).

The ArrayTube^{TM} microarray system is also relatively inexpensive, when hands-on-time, necessary equipment, and time are considered. It can be carried out automatically, as it is

based on a simple spot pattern recognition assay and had very high throughput and a short turnaround time when compared to the other molecular typing methods assessed here. The multistep method, DNA microarray analysis (includes amplification, labeling, hybridization etc.) is not technically difficult. The results of the analysis are given as images and bar-plot diagrams where positive and negative hybridization signals are differentiated at a value of 0.3 (Figure 3.5). Data analysis of microarray results is a simple computerized step. The whole procedure using the AT^{TM} system is an extremely portable process which needs only a minimum of standardization (Miller and Tang, 2009; Buchanan et al., 2011). A further benefit of the microarray assay is the integration of the whole genome in the investigation instead of only one or a limited number of genes.

In general, a method that yields discrimination values of higher than 0.95 can be considered more or less "ideal" (Behringer et al., 2011). However, selection of the typing method depends upon many variables such as cost, difficulty of technique performance, and interpretation of results. On the basis of discriminatory power, DNA microarrays (D = 1.00) appear to be the preferred method used for typing of *C. jejuni* through routine surveillance.

In summary, it was shown that different typing methods reveal the same results: a genetic heterogeneity of *Campylobacter* isolates from turkey during the rearing process was observed, but the degree of relatedness was different depending upon the typing method. Use of more than a single method gives clarity about the genetic heterogeneity within the *Campylobacter* population.

The *C. jejuni* isolates were shown to be genetically stable during 20 *in vitro* passages corresponding with findings of others (Ayling et al., 1996; Meinersmann et al., 1997; Wassenaar et al., 1998; Ridley et al., 2008). However, previous reports also described genomic instability among campylobacters (Harington et al., 1997; Hänninen et al., 1999; Wassenaar and Newell, 2000).

In the present study, the genetic diversity among *C. jejuni* isolates was investigated in a single turkey flock. The turkeys harbored more than one genotype of *C. jejuni* in the same rearing cycle. The investigation demonstrated clearly a dynamic in changing of the *Campylobacter* population in turkeys as well as a coexistence of different genotypes for the first time, to our knowledge, during turkey rearing. The heterogeneity profiles differed according to the typing methods in which DNA microarray-based comparative genomic hybridization analysis provides the most powerful alternative for *C. jejuni* genotyping. Based upon the results of genotyping and *in vitro* stability tests, it seems clear that different strains had colonized the turkeys at different dates, alongside the rearing process. The sources for *Campylobacter* transmission into the turkey flock are not yet known.

6. Acknowledgments

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C H A P T E R 4

Antimicrobial sensitivity in *C. jejuni* in turkey farms in Germany

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Determination of Antimicrobial Sensitivities of *Campylobacter jejuni* Isolated from Commercial Turkey Farms in Germany

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1. Summary

The emergence of antimicrobial resistance among *Campylobacter* isolates recovered from turkeys has increased dramatically. Monitoring the progress of this resistance becomes a growing public health issue. The aim of the present study was to provide information of the current status of antibiotic resistance patterns in *Campylobacter jejuni* from turkeys. Seventy-six *C. jejuni* isolates were recovered from 67 epidemiologically unrelated meat turkey flocks in different regions of Germany in 2010 and 2011. The isolates were typed by *fla*A genotyping and were investigated for antimicrobial susceptibility against 12 antibiotics by using a broth microdilution test as well as testing the genetic determination of ciprofloxacin, tetracycline, and erythromycin resistance. All isolates (n = 5 76) were sensitive to gentamicin and chloramphenicol. The numbers of isolates that were sensitive to streptomycin, erythromycin, neomycin, and amoxicillin were 69 (90.8%), 61 (80.2%), 58 (76.4%), and 44 (57.9%), respectively. Only one isolate was sensitive to all tested antibiotics. The emergence of a high resistance rate and multidrug resistance to three or more classes of antimicrobial agents were observed. The resistance against sulphamethoxazole/trimethoprim, metronidazole, ciprofloxacin, naladixic acid, and tetracycline was 58 (76.3%), 58 (76.3%), 53 (69.7%), 51 (67.1%), and 42 (55.3%), respectively. None of the isolates was resistant to all antibiotics. Multidrug resistance to three or more classes of antimicrobial agents was found and ranged from 3.9% to 40.8%. Replacement of the Thr-86→Ile in *gyr*A gene and detection of the *tet*(O) gene were the main resistance mechanisms for fluoroquinolones and tetracycline, respectively, while the lack of mutation in position 2074 and 2075 on the 23S *rRNA* gene was responsible for macrolide resistance. The phenotypic and genotypic resistance profiles were compatible in the case of ciprofloxacin and tetracycline but were not completely congruent with respect to erythromycin.

http://dx.doi.org/10.1637/10135-031912-Reg.1

C H A P T E R 5

Turkey as a new avian host for *Ochrobactrum* species

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Isolation and characterization of *Ochrobactrum anthropi* and *Ochrobactrum pecoris* from caecal content of commercial turkeys

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1. Summary

Two Gram negative, micro-aerophilic, non-motile and non-spore-forming coccoid bacteria were isolated from female turkey caecal samples collected from a slaughterhouse. The biochemical reaction profiles (API 20 E and API 20 NE) typed both strains as *Ochrobactrum anthropi*. On the basis of 16S rRNA gene and *rec*A gene sequence similarities the strains were identified as *Ochrobactrum anthropi and Ochrobactrum pecoris*, respectively. Both strains were highly resistant against beta-lactam antibiotics, chloramphenicol and sulphonamides but variable in susceptibility to ciprofloxacin, gentamicin and tetracycline. This is the first time that *Ochrobactrum* species were isolated from an avian host, i.e. turkey.

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Summary of the thesis

Summary

Poultry remains an important vehicle for pathogens, leading to human food borne disease all over the world. *Campylobacter* infection in commercial broilers and turkeys represents a challenge for all persons involved in poultry food production chain. Studying the colonization and genetic diversity along with the antimicrobial resistance of *Campylobacter jejuni* in commercial poultry rearing is a matter of concern. The findings of this thesis clearly indicate that age and -possible- sex have an influence on the onset of colonization and prevalence of thermophilic *Campylobacter* in fattening turkey farms. Drinking water can be considered as primary source for flock infection. Additionally, multiplex PCR used for detection of thermophilic *Campylobacter* DNA which was directly extracted from faecal and environmental samples was developed and evaluated (Chapter 2).

The assessment of the genetic diversity of a *Campylobacter* population is critical for the understanding of the epidemiology of this bacterium and consequently this information has to be used to reduce disease burden. Hence, a part of this study was conducted to investigate the dynamic of flock colonization and genetic diversity in *Campylobacter* isolates using different genotyping tools. The performance of established *fla*A genotyping, multilocus sequencing typing (MLST) and DNA microarray typing assay based on the ArrayTube™ technology was evaluated using *C. jejuni* isolates that proved to be genetically stable in their *fla*A genes. It was shown that different typing methods are useful to determine the genetic heterogeneity of *C. jejuni* isolated from turkey during the rearing process. The degree of relatedness was varying depending upon the typing method used. DNA microarray technique based on whole genome information had the highest discriminatory power compared to the other molecular typing methods assessed here. The AT^{TM} microarray system is also relatively cheap, when hands-on-time, necessary equipment, and costs of material are considered. This investigation demonstrated changing of *C. jejuni* in turkeys and the coexistence of different genotypes during turkey rearing for the first time (Chapter 3).

A recent concern is the emergence of antibiotic resistance in *C. jejuni* isolated from turkeys in particular of those antibiotics used to treat human illness. Monitoring the progress of this resistance becomes a growing public health issue. The third part of this study was conducted to provide information about the resistance of *C. jejuni* isolated from turkeys by investigating the genes for ciprofloxacin, tetracycline and erythromycin resistance. All isolates were fully susceptible to chloramphenicol and gentamicin. The isolates were highly resistant to

sulphonamides, metronidazole, ciprofloxacin, naladixic acid, and tetracycline. The isolates were sensitive to streptomycin, erythromycin, neomycin, and amoxicillin. Multidrug resistance was detected. Replacement of the Thr-86→Ile of the *gyr*A gene, and a *tet*(O) gene were the main resistance mechanisms for fluoroquinolones and tetracycline resistance, respectively, while no point mutation in the 23S rRNA gene was found that could be responsible for macrolide resistance. The present study provides sufficient data suggesting that the PCR-RFLP and MAMA-PCR are simple and rapid methods for the detection of ciprofloxacin resistance in *C. jejuni*. These methods seem to be suitable to serve as possible alternative methods for routine detection of mutations without the need for sequencing. To our knowledge, this is the first study providing sufficient data on the current status of the antimicrobial susceptibility to *C. jejuni* isolated from turkey farms in different regions in Germany (Chapter 4).

During the trail to isolate *Campylobacter* from caecal content of female turkey at 16th week of age Two Gram negative, micro-aerophilic, non-motile and non-spore-forming coccoid bacteria were isolated. The biochemical reaction profiles (API 20 E and API 20 NE) typed both strains as *Ochrobactrum anthropi*. On the basis of 16S rRNA gene and *rec*A gene sequence similarities the strains were identified as *Ochrobactrum anthropi* and *Ochrobactrum pecoris*, respectively. Both strains were highly resistant against beta-lactam antibiotics, chloramphenicol and sulphonamides but variable in susceptibility to ciprofloxacin, gentamicin and tetracycline. This is the first time that *Ochrobactrum* species were isolated from an avian host (Chapter 5).

Zusammenfassung

Thermophile *Campylobacter* aus Puten in Deutschland
Epidemiologie, Genotypisierung und Antibiotika-Empfindlichkeits untersuchungen

Geflügel bleibt ein wichtiges Vehikel für Krankheitserreger, die zu menschlichen Lebensmittel übertragen Krankheit auf der ganzen Welt. *Campylobacter*-Infektion bei kommerziellen Masthähnchen und Puten stellt eine Herausforderung für alle Personen in Geflügel Nahrungsmittelproduktionskette beteiligt.

Studien zur Kolonisierung, genetischen Vielfalt sowie der Resistenzentwicklung von *Campylobacter jejuni* in Nutzgeflügel sind deshalb von großer Wichtigkeit. Die Ergebnisse dieser Studie zeigen deutlich den Einfluss von Alter und – möglich - Geschlecht von Puten auf den Beginn der Kolonisation und die Prävalenz von thermophilen *Campylobacter*.

Tränkenwasser kann als primäre Quelle für Herde Infektion in Betracht gezogen werden. Zusätzlich Multiplex-PCR zum Nachweis von thermophiler *Campylobacter* DNA, die direkt von Fäkal-und Umweltproben wurde extrahiert verwendet wurde entwickelt und evulated (Kapitel 2).

Die Beurteilung der genetischen Vielfalt von *Campylobacter*-Isolaten ist von entscheidender Bedeutung für das Verständnis der Epidemiologie dieses Bakteriums. Die so gewonnenen Daten helfen auch, die Durchseuchung der Bestände und die Inzidenz zu verringern. Daher wurde eine Studie durchgeführt, um die Dynamik der Besiedlung eines Bestandes zu untersuchen und die genetische Vielfalt der isolierten *Campylobacter* mit verschiedenen Genotypisierungsmethoden zu ermitteln. Die Qualität der etablierten *fla*A-Genotypisierung, Multilocus-Sequenz-Typisierung (MLST) und DNA-Mikroarray-Testung mittels der ArrayTube™-Technologie wurde mit *fla*A-Gen stabilen *C. jejuni*-Isolaten durchgeführt. Es konnte gezeigt werden, dass alle Typisierungsmethoden eine größere genetische Heterogenität der Puten-Isolate während der Aufzucht wiedergaben, die ermittelten Verwandtschaftsverhältnisse der Isolate aber abhängig von der Untersuchungsmethode waren. Die DNA-Mikroarray-Analyse, welche das gesamte Genom in die Untersuchung einbezieht, hatte die höchste Trennschärfe im Vergleich zu den anderen molekularen Typisierungsmethoden. Das AT^{TM} Mikroarray-System ist preiswert, wenn Zeitaufwand, notwendige Ausrüstung und die Materialkosten berücksichtigt werden. Die Untersuchung ergab eine dynamische Änderung der *C. jejuni*-Population in den Puten, wobei auch ein

gleichzeitiges Nebeneinander verschiedener Genotypen während der Aufzucht feststellbar war (Kapitel 3).

Besorgniserregend ist in den letzten Jahren die Entstehung von Antibiotika-Resistenzen bei *C. jejuni*-Puten isolaten, insbesondere für solche Antibiotika, die verwendet werden, um humane Erkrankungen zu behandeln. Die Überwachung der Resistenzausbildung wird ein wachsendes Problem des öffentlichen Gesundheitswesens. Daher wurde der dritte Teil dieser Studie durchgeführt, um aktuelle Informationen über die Antibiotikaresistenzen in *C. jejuni*-Puten-isolaten zu erhalten. Dazu wurden auch molekularbiologische Untersuchungen zur Ciprofloxacin-, Tetracyclin- und Erythromycin-Resistenz durchgeführt. Alle Isolate waren empfindlich gegenüber Chloramphenicol und Gentamicin. Die Isolate waren hochgradig resistent gegen Sulfonamide, Metronidazol, Ciprofloxacin, Nalidixinsäure und Tetracyclin. Viele Isolate waren empfindlich gegenüber Streptomycin, Erythromycin, Neomycin und Amoxicillin. Ebenso konnten Mehrfachresistenzen bei verschiedenen Isolaten nachgewiesen werden. Ersatz des Thr-86 → Ile im *gyr*A-Gen und die Detektion des *tet*(O)-Gens waren die wichtigsten Resistenzmechanismen für Fluorchinolone und Tetracyclin, Resistenz während keine Punktmutation in der 23S-rRNA-Gen gefunden wurde, die für die Makrolidresistenz verantwortlich gemacht wird. Die vorliegende Studie liefert ausreichend Daten dafür, dass die PCR-RFLP und PCR-MAMA einfache und schnelle Verfahren zum Nachweis der Ciprofloxacin-Resistenz in *C. jejuni* darstellen und als Alternative für den routinemäßigen Nachweis der Mutation, ohne die Notwendigkeit einer DNA-Sequenzierung dienen können. Die Studie stellt erstmals seit Jahren den aktuellen Status der antimikrobiellen Empfindlichkeit für *C. jejuni*-Isolate aus Putenhaltungen verschiedener Regionen Deutschlands dar (Kapitel 4).

Während der Weg zum *Campylobacter* aus Blinddarm Inhalte der weiblichen Mastputen zu isolieren, die am 16. Woche im Alter von zwei gramnegative wurden mikroaerophilen, unbewegliche und nicht Sporen bildende Kokken isoliert. Die biochemische Reaktion Profile (API 20 E und API 20 NE) eingegeben beide Stämme als *Ochrobactrum anthropi*. Auf der Basis der 16S-rRNA-Gen und *rec*A-Gen-Sequenz Ähnlichkeiten wurden die Stämme als *Ochrobactrum anthropi* und *Ochrobactrum pecoris* identifiziert sind. Beide Stämme waren resistent gegenüber beta-Lactam-Antibiotika, Chloramphenicol und Sulfonamiden, aber unterschiedlich in ihrer Empfindlichkeit gegenüber Ciprofloxacin, Gentamicin und Tetracyclin. Dies ist das erste Mal, dass *Ochrobactrum* von einem aviären Wirt isoliert werden konnte. (Kapitel 5).

List of publications

A) Publications in peer-reviewed journals

1- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Taboada EN, Ehricht R, Hafez HM **(2013)** Detection of genetic diversity in *Campylobacter jejuni* isolated from a commercial turkey flock using *fla*A typing, MLST analysis and microarray assay. PLoS ONE 8(2):e51582.

2- **El-Adawy H,** Hotzel H, Düpre S, Tomaso H, Neubauer H, Hafez HM **(2012)** Determination of antimicrobial sensitivities of *Campylobacter jejuni* isolated from commercial turkey farms in Germany. Avian Dis. 56:685–692.

3- Schilling AK, Hotzel H, Methner U, Sprague LD, Schmoock G, **El-Adawy H**, Ehricht R, Wöhr AC, Erhard M, Geue L. **(2012)** Zoonotic agents in small ruminants kept on city farms in southern Germany. Appl. Environ. Microbiol. 78:3785-3793.

4- **ElAdawy H**, Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Isolation and characterization of *Ochrobactrum anthropi* and *Ochrobactrum pecoris* from caecal content of commercial turkeys. Vet. Microbiol. 155:349–354.

5- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Elucidation of colonization time and prevalence of thermophilic *Campylobacter* species during turkey rearing using multiplex PCR. Poultry Sci. 91:454-459.

6- Kiehntopf M, Melcher F, Hänel I, **Eladawy H**, Tomaso H **(2011)** Differentiation of *Campylobacter* species by surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry. Foodborne Pathog. Dis. 8:875-885.

The papers 1, 2, 4, and 5 in have evolved in my doctoral thesis.

B) Publications in academic conferences

1- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2013)** Molecular detection of antibiotic resistance in *Campylobacter jejuni* isolated from turkey flocks in Germany. 7th "Hafez" International Symposium on Turkey Production Berlin, Germany 30th Mai – 1st June 2013 (In press).

2- **El-Adawy H,** Marwa F. E. Ahmed, Hotzel H, Tomaso H, Neubauer H, Hartung J, Hafez HM **(2013)** Investigation and characterization of *Campylobacter* in free range turkeys . th "Hafez" International Symposium on Turkey Production Berlin, Germany !: th Mai – 1st June 2013 (In press).

3- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Turkey as a new host for *Ochrobactrum* species. In Proc. 9th International Symposium on Turkey Diseases, ISBN: 978-3-86387-254-0. Ed. by H. M. Hafez. 21stM&!rd June 2012, Berlin, Germany, 91-98.

4- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Evaluation of genotyping methods for detection of genetic diversity among *C. jejuni* strains isolated from a turkey flock. In Proc. 9th International Symposium on Turkey Diseases, ISBN: 978-3-86387-254-0. Ed. by H. M. Hafez, 21stM&!rd June 2012, Berlin, Germany, 136-147.

5- **El-Adawy H,** Hotzel H, Düpre S, Tomaso H, Neubauer H, Hafez HM **(2012)** Untersuchung zur Antibiotika-Resistenz von *Campylobacter jejuni*-Isolaten deutscher Puten. In Proc. DVG-Tagung der Fachgruppe "Bakteriologie und Mykologie" in Verbindung mit dem Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig ISBN: 978-3-86345-080-9. 27th +&2th June 2012, Leipzig, 85-86.

6- Schilling AK, Hotzel H, Methner U, Sprague LD, Schmoock G, **El-Adawy H**,Ehricht R, Wöhr AC, Erhard M, Geue L. **(2012)** Zoonosen-Erreger bei auf Jugendfarmen und verwandten Einrichtungen in Bayern und Baden-Württemberg gehaltenen Kleinen Wiederkäuern. In Proc. DVG-Tagung der Fachgruppe "Bakteriologie und Mykologie" in Verbindung mit dem Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig ISBN: 978-3-86345-080-9. 27th+ &2th June 2012 Leipzig, 144-145.

7- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2011)** Multiplex PCR for detection of *Campylobacter* DNA during commercial turkey production. In Proc. , th International Symposium on Turkey Production, ISBN: 978-3-86387-094-2, Ed.by H. M. Hafez, 16th -18th June 2011, Berlin, Germany, 261-266.

C) Oral presentations

1- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2013)** Molecular detection of antibiotic resistance in *Campylobacter jejuni* isolated from turkey flocks in Germany. . th "Hafez" International Symposium on Turkey Production Berlin, Germany 30th Mai – st June 2013 (English language).

2- **El-Adawy H,** Marwa F. E. Ahmed, Hotzel H, Tomaso H, Neubauer H, J. Hartung, Hafez HM **(2013)** Investigation and characterization of *Campylobacter* in free range turkeys . th

"Hafez" International Symposium on Turkey Production Berlin, Germany 30th Mai – 1st June 2013 (English language).

3- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Turkey as a new host for *Ochrobactrum* species. 9th International Symposium on Turkey Diseases, 21stM &!rd June 2012, Berlin, Germany (English language).

4- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2012)** Evaluation of genotyping methods for detection of genetic diversity among *C. jejuni* strains isolated from a turkey flock. 9th International Symposium on Turkey Diseases, 21st– 23rdJune 2012, Berlin, Germany (English language).

5- **El-Adawy H (2011)** *Campylobacter* bei Mastputen, Wissenschaftliches Seminar der DVG-Fachgruppe „Bakteriologie und Mykologie" in Zusammenarbeit mit der Fachgruppe Zoonosen der DGHM und dem Friedrich-Loeffler-Institut (FLI), 24th October 2011, Jena, Deutschland (German language).

6- **El-Adawy H** and Hotzel H **(2011)** Antimicrobial susceptibility testing of German *Campylobacter jejuni* isolates originated from turkeys. Minisymposium "Antibiotic resistome in the food chain", 10 thM th October 2011, Jena, Germany (English language).

7- **El-Adawy H,** Hotzel H, Tomaso H, Neubauer H, Hafez HM **(2011)** Multiplex PCR for detection of *Campylobacter* DNA, 6th International Symposium on Turkey Production, , th+ 0th June 2011, Berlin, Germany (English language).

D) Posters

1- **El-Adawy H,** Hotzel H, Düpre S, Tomaso H, Neubauer H, Hafez HM **(2012)** Untersuchung zur Antibiotika-Resistenz von *Campylobacter jejuni*-Isolaten deutscher Puten. DVG-Tagung der Fachgruppe "Bakteriologie und Mykologie" in Verbindung mit dem Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig. 27th+&2th June 2012, Leipzig, Germany.

2- Schilling AK, Hotzel H, Methner U, Sprague LD, Schmoock G, **El-Adawy H,** Ehricht R, Wöhr AC, Erhard M, Geue L **(2012)** Zoonosen-Erreger bei auf Jugendfarmen und verwandten Einrichtungen in Bayern und Baden-Württemberg gehaltenen kleinen Wiederkäuern. DVG-Tagung der Fachgruppe "Bakteriologie und Mykologie" in Verbindung mit dem Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig. 27th+&2th June 2012, Leipzig, Germany.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 19.07.2013 Hosny Hassan Hosny El-Adawy