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Abbreviations

*asp*A	Aspartase A gene
AT	ArrayTube
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
EFSA	European Food Safety Authority
EU	European Union
*fla*A	Flagellin A gene
*fla*B	Flagellin B gene
FQ	Fluoroquinolone
GDP	Gross Domestic Product
*gln*A	Glutamine synthetase gene
*glt*A	Citrate synthase gene
*gly*A	Serine hydroxymethyl transferase gene
GBS	Guillain-Barré syndrome
*gyr*A	Gyrase A gene
hip	Hippurate gene
HIV	Human immunodeficiency virus
Ile	Isoleucine
ISO	International Standard Organization
MAMA-PCR	Mismatch amplication mutation assay polymerase chain reaction
*map*A	Outer membrane lipoprotein gene
mCCDA	Modified charcoal cefaperazone deoxycholate agar
MIC	Minimum inhibitory concentration

MLST	Multilocus sequence typing
mPCR	Multiplex polymerase chain reaction
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PFGE	Pulsed field gel electrophoresis
pgm	Phosphoglucomutase gene
pH	pH value - measure of the activity of hydrogen ions
qrt-PCR	Quantitative real time PCR
QRDR	Quinolone resistance determining region
*rec*A	Recognase A gene
RFLP	Restriction fragment length polymorphism
ST	Sequence type
tet(O)	Tetracycline resistance gene (O)
Thr-86-Ile	Threonine to isoleucine mutation
tkt	Transketolase gene
UK	United Kingdom
*unc*A	ATP synthase alpha subunit gene
VBNC	Viable but nonculturable colony

Introduction

A zoonosis is an infectious disease that can be transmitted from animals to humans and *vice versa*. *Campylobacter* (*C.*) are examples for zoonotic agents. Thermophilic *Campylobacter* species have become the most frequent cause of bacterial gastroenteritis in humans worldwide (ECDC, 2014). Infections by *Campylobacter* exceed the total number of those caused by *Salmonella*, *Shigella*, and *Escherichia coli* O157:H7 in humans (EFSA, 2011; ECDC, 2014). Campylobacteriosis is normally self-limiting in adults but can cause diarrhoea or even mortality in children in developing as well as in developed countries. Poultry and poultry products are an important vector for transmission of *Campylobacter* to humans (Hafez, 2003). The natural habitat of thermophilic *Campylobacter* is the intestinal tract of birds. Most commonly, transmission to humans occurs through consumption and handling of chicken meat products which were contaminated during the slaughtering process.

Vietnam is an agricultural and developing country located in the Southeast Asia with > 70% of the population living in rural areas (Vang, 2003; Hanh et al., 2007). Agricultural production contributed 20.9% of Gross Domestic Product (GDP). The productive value of poultry ranks second (341.9 million heads) in husbandry industry after pig production, and accounted for 19% of the total livestock production. Vietnam also ranks 5th in ASEAN and 47th in the world in poultry meat and egg yield production (Truc, 2011). Although poultry consumption is of great importance in Vietnam threats for human health caused by foodborne zoonoses like campylobacteriosis limited warnings official authorities have launched only.

Vietnam is a so-called hotspot for emerging infectious diseases in Southeast Asia (Coker et al., 2011). Nevertheless, infections with thermophilic *Campylobacter* have been frequently neglected. Previous studies were focused mostly on prevalence of *Campylobacter* in children (Bodhidatta et al., 2007; My et al., 2013). Also the prevalence of intestinal bacterial pathogens in adults was investigated (Trang et al., 2007) and *Campylobacter* was detected in 31% of the study population. Fifteen to 32% of meat samples in different regions of Vietnam contained thermophilic *Campylobacter* (Carrique-Mas et al., 2013; Huong et al., 2006; Ha and Pham, 2006; Schwan, 2010; Garin et al., 2012; Carrique-Mas et al., 2014). Duck and pork meat were contaminated with *Campylobacter* in 23.9% and 53.7% of tested samples, respectively (Carrique-Mas et al., 2014). Bao et al. (2006) isolated thermophilic campylobacters from 35.1% of chicken carcasses in large and small abattoirs of Ho Chi Minh City. Nearly 70% of

the isolates belonged to the species *C. jejuni* (Bao et al., 2006). However, molecular characterization of *Campylobacter* from Vietnam is still limited.

Kenya is also a developing country located in East Africa where agriculture contributes 25% of GDP. Poultry production is playing a major role, representing 30% of the agricultural contribution to the GDP (Okeno et al., 2012; Mwobobia et al., 2016). The country has a total of 37.3 million chickens with 90% of these kept by rural farmers in traditional extensive backyards as an important economic activity (Nyaga, 2007; Mwobobia et al., 2016). Antibiotics are used extensively in poultry production in Kenya for treatment, prevention of diseases, promotion of growth and to improve feed efficiency. This practice is believed to contribute to the development and spread of antibiotic resistance in bacteria.

In general, the knowledge about *Campylobacter* in Kenya is limited. A study from western Kenya showed that 20% of people with diarrhoea were infected by *Campylobacter* but in the group of healthy children below 5 years *Campylobacter* was detected in 42% (Brooks et al., 2006). Other studies reported *Campylobacter* as cause of foodborne diseases (Ombui et al., 2001) and contamination of raw chicken and beef from butcheries and markets in Nairobi as a major source was reported (Osano and Arimi, 1999). Resistance against antibiotics in bacteria is of public health concern. Kenyan *Campylobacter* isolates from humans showed a high resistance rate to erythromycin (52%), but in the past only low resistance to ciprofloxacin, tetracycline and nalidixic acid with 6%, 18% and 26%, respectively was found (Brooks et al., 2006). Awareness of an increase of antibiotic resistance in *Campylobacter* is rising and warnings have been launched not to misuse antibiotics like macrolides, fluoroquinolones or alternative drugs (EFSA, 2015).

Studies on the epidemiology of *Campylobacter* based on molecular techniques are limited for both countries. Additionally, some common characteristics of chicken production and food consumption habits are in common, Vietnam and Kenya, e.g. close proximity of human and animal production, low biosecurity levels and hygiene standards, overuse of antibiotics in animal feed. These factors have been discussed to contribute to *Campylobacter* transmission to human. There are many gaps that need to be filled in epidemiological studies of *Campylobacter* in these countries. Therefore, the aim of this work is phenotyping and genotyping of *Campylobacter* isolates from Vietnam and Kenya and to elucidate antimicrobial resistance of *Campylobacter* isolates. Specifically, this study focusses on:

1. Giving an overview on campylobacteriosis as a neglected infection in Southeast Asia.
2. Genotyping and detection of antibiotic resistance of thermophilic *Campylobacter* isolated from chicken and pig meat in Vietnam.
3. Determining the antibiotic resistance of *Campylobacter* isolates cultivated from small scale and backyard chicken in Kenya.

CHAPTER 1

Review of Literature

Review of the literature

1. *Campylobacter*

1.1. The organism

In 1886, Escherich investigated stool samples of children that had died from diarrhea and found organisms with curved shape with his microscope which resembled bacteria of the genus *Vibrio* (*V.*) (Kist, 1986; Debruyne et al., 2008; Vandamme et al., 2010). He found similarities in cell morphology and biochemical characteristics between them. Unfortunately, his report of results was published in German language in the Münchener Medizinische Wochenschrift and was ignored for a long time. Additionally, cultivation and isolation of this kind of bacteria at that time was not possible and limited further studies on the microorganisms.

Until 1909, *Campylobacter* was isolated from aborted fetuses of ewes by two veterinary surgeons (McFadyean and Stockman, 1913). In 1919, these bacteria were described as a *Spirillum* species by Smith and Taylor when isolated from aborted bovine fetuses. Therefore, the species was named "*Vibrio fetus*". Between 1931 and 1944, isolates were recovered from calves and also pigs with dysentery and were named *V. jejuni* (Jones et al., 1931; Doyle, 1944).

Campylobacter was found in a wide range of animals at that time. This caused confusions regarding the taxonomy for *Vibrio* species because they were found in diverse habitats e.g. the bovine and ovine genital tract, the human oral cavity, and bovine intestinal contents.

In 1963, the result of determination of the G+C ratio in genomic DNA showed that *V. fetus* and *V. bubulus* were notably different from other *Vibrio* species. Thus, *Campylobacter* was proposed as a new genus (Sebald and Veron, 1963). More comprehensive taxonomic studies later on used various biochemical and serological tests and the G+C ratio in DNA. *Vibrio* bacteria were reclassified as *Campylobacter* with a greater level of acceptance in the scientific community (Veron and Chatelain, 1973).

Campylobacter belong to the delta epsilon class of proteobacteria and form together with the genera *Arcobacter* and *Sulfurospirillum* the family *Campylobacteraceae* within the order of *Campylobacterales*.

Currently, the genus *Campylobacter* comprises more than 20 species and 6 subspecies (phylogenetic relationships) based on 16S rRNA gene sequence comparisons (Table 1).

Table 1. Taxonomy of *Campylobacter*

Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Epsilon proteobacteria
Order	*Campylobacterales*
Family	*Campylobacteraceae*
Genus	*Campylobacter*
Species	*C. jejuni*, *C. coli*, *C. concisus*, *C. curvus*, *C. faecalis*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis*, *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. upsaliensis* and others
Subspecies	*C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. hyointestinalis* subsp. *lawsonii*, *C. jejuni* subsp. *doylei*, *C. jejuni* subsp. *jejuni*

However, several species still remain a matter of controversy and require further investigation. The species *Bacteroides* (*B.*) *ureolyticus* is an interesting example. Using the 16S rRNA gene analysis, it was clearly demonstrated that *B. ureolyticus* was more closely related to *Campylobacter* than to *Bacteroides* spp. Nonetheless, fatty acid content, disease caused, proteolytic metabolism and urease production of *B. ureolyticus* are atypical for *Campylobacter* spp. (Vandamme, 2000). It is therefore emphasized that its taxonomic position requires clarification through further studies.

Campylobacter spp. are microaerophilic growing and non-spore forming Gram negative bacteria. *Campylobacter* grow as flat and greyish or smooth and glossy colonies on solid media (Karmali and Skirrow, 1984). They show in investigations by electron microscopy a spiral or S-shape form but most common is the coccoid form (Lai-King et al., 1985). The Greek word “kampylos” means S shape. The form is slender. The size is from 0.5 µm to 8µm in length and 0.2µm to 0.5µm in wide. Darting or rapid motility facilitated by unsheathed polar flagella at one or both ends of the cells is characteristic for *Campylobacter* and is used to differentiate them from other enteric organisms (Karmali and Skirrow, 1984).

An important adaption of *Campylobacter* to unfavourable conditions or stress is their ability to fall into a viable but non-culturable (VBNC) state. When this state was formed, it resulted

in a change of cell morphology from the usual spiral shaped rod to a coccoid form leading to decreased metabolic activity. The mechanisms are not entirely unclear due to the inability to detect these bacteria by conventional culture techniques.

1.2. Viable but non-culturable *Campylobacter* (VBNC)

The viable but non-culturable state of thermophilic *Campylobacter* has been recorded for last two decades (Rollins and Colwell, 1986; Tholozan et al., 1999). *Campylobacter* VBNC is connected with very low metabolic activity under unfavourable growth conditions such as adverse nutrients, temperature, oxygen or light conditions (Oliver, 2005). Standard culture methods cannot detect VBNC cells efficiently. Temperature is a very important factor for survival. It was shown that cultured cells were dead within 3 days when the cells were incubated at 25°C (Medema et al., 1992). *C. jejuni* cells incubated at 25°C or 37°C metabolized nutrients faster than cells incubated at 4°C (Jones et al., 1991). The VBNC forms of some *C. jejuni* strains were enumerated and characterized by using 5-cyano-2,3-ditolyl tetrazolium chloride–4′,6-diamino-2-phenylindole staining (Tholozan et al., 1999).

1.3. Growth requirements

Thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*) grow under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) and temperatures between 37°C and 42°C. Optimal growing temperature of thermophilic *Campylobacter* spp. is 41.5°C. Thermophilic *Campylobacter* spp. are incapable of growing below 30°C or at a pH value below 4.9 or above 9.0 (Hazeleger et al., 1995). They are inactivated at -15°C but survive at 4°C (Hazeleger et al., 1995). Freezing conditions reduce the population of *Campylobacter* (Stern and Kotula., 1982). High temperate requirements may reflect an adaption to the intestines of birds. In routine laboratories, cultivation of *Campylobacter* is carried out on selective media e.g. modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) or Mueller Hinton agar distinguishing them from other enteric bacteria.

1.4. Isolation of thermophilic *Campylobacter*

Isolation of thermophilic *Campylobacter* is performed in accordance with the official protocol of the International Standards Organization (ISO) 10272-1. It is applied widely and needs 4 days to give negative results and 6-7 days to confirm a positive result. The procedure consists of two steps: enrichment and cultivation on selective agar. For the enrichment Bolton broth or Preston broth are recommended (Rogol et al., 1985). Typically, samples are incubated in

enrichment medium at 37°C for 4-6 h and then at 42°C for two days at/under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). As selective media Skirrow agar (Skirrow, 1977), Preston agar (Bolton and Robertson, 1982), 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) can be used. Selective media are commonly prepared with sheep blood. Additionally, these selective media use antibiotic supplements to inhibit the growth of *Enterobacteriaceae*, enterococci, fungi etc. Cephalosporins are commonly used in combination with other antibiotics such as trimethoprim, vancomycin, amphotericin, rifampicin (Corry et al., 1995). Cultivation is done at 37°C and 42°C for 48 h at/under microaerophilic atmosphere.

However, usage of a membrane filter has also been recommended for isolation of *Campylobacter* from faeces. It can be an alternative to usage of antibiotic supplements, especially for highly sensitive agents like *C. upsaliensis* (Lopez et al., 1998).

1.5. Detection and differentiation of *Campylobacter*

Conventional detection methods are based on cultural methods and were combined with biochemical tests to identify and differentiate *Campylobacter* spp. (On and Holmes, 1991a; On and Holmes, 1991b; On and Holmes, 1992; Ugarte-Ruiz et al., 2012). *C. jejuni* and *C. coli* grow on the same selective agars, therefore it is necessary to differentiate them. This can be done on the basis of hippurate hydrolysis (OIE, 2008). *C. jejuni* shows hydrolysis of hippurate, whereas *C. coli* is not able to hydrolyze sodium hippurate. However, unclear results on the basis of phenotypic characteristics make identification of *Campylobacter* spp. difficult. Alternative procedures have been developed for rapid detection, differentiation and confirmation of *Campylobacter* spp., including *in situ* hybridization (Lehtola et al., 2006), latex agglutination (Hazeleger et al., 1992), enzyme immunoassay (Endtz et al., 2000), and nucleic acid-based methods like polymerase chain reaction (PCR) (Denis et al., 1999).

The polymerase chain reaction was invented in 1985 to amplify specific segments of DNA. Species specific PCR applications were an important step forward in the detection of *Campylobacter* spp. An example is the specific detection of *C. jejuni* based on the presence of the hippurase gene (Slater and Owen, 1997). However, PCR assays may give false-negative results (Steinhauserova et al., 2001). First PCR assays for the identification of *C. jejuni* and *C. coli* were based on 16S and 23S rRNA gene sequences (Giesendorf et al., 1992; Eyers et al., 1993). However, it was noted that species like *C. jejuni* and *C. coli* share identical or nearly

identical 16S rRNA gene sequences. (On, 2005). Sequence similarity up to 98% of *C. lari* and *C. jejuni* has also been reported for the internal spacer regions of the rRNA operons (Miyajima et al., 2002).

Besides conventional PCR technique for detection of *Campylobacter* spp. multiplex PCR (mPCR), and quantitative real-time PCR (qrt-PCR) have also been developed. Discrimination using these assays between *C. jejuni* and *C. coli* was described (Gonzalez et al., 1997; Linton et al., 1997; Lawson et al., 1998). A mPCR assay was also developed by Denis et al. (1999) for simultaneous discrimination of *C. jejuni* and *C. coli* . This assay was widely applied. El-Adawy et al. (2012) developed a mPCR system to detect *C. jejuni*, *C. coli* and *C. lari* simultaneously. Meanwhile, the application of PCR techniques for detection and differentiation of *Campylobacter* spp. has become popular and available in many laboratories due to its efficacy and low costs.

1.6. Genotyping of thermophilic *Campylobacter*

Typing methods are flagellin typing, multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP) and microarray analysis.

1.6.1. Flagellin typing

A key feature of many pathogenic bacteria is their motility by means of flagella (Jagannathan and Penn, 2005). Both *C. jejuni* and *C. coli* have two genes, *fla*A and *fla*B, which are responsible for the expression of the flagellar filament. The *fla*A is the major and *fla*B is the minor component of the flagellum, however, not in all cases these genes were expressed simultaneously (Wassenaar et al., 1993). The sequence of *fla*A showed a higher heterogeneity than the *fla*B gene (Fischer and Nachamkin, 1991). The presence of highly conserved and variable regions in *fla*A genes makes this locus highly suitable for PCR-RFLP analysis (Shi et al., 2002). Thus, *fla*A typing is usually applied to examine the diversity among *Campylobacter* isolates. The *fla*A typing is a combination of two techniques: PCR and restriction fragment length polymorphism (RFLP), in which the PCR aims to amplify a larger part of the *fla*A gene and then these amplicons are analyzed using restriction fragment length polymorphism (RFLP) to determine mutations of the genes. PCR-restriction fragment length polymorphism (PCR-RFLP) is a PCR-based method for detecting polymorphisms in DNA fragments. Amplification is followed by digestion of amplicons with restriction enzymes. Depending on cutting sites in amplicons different patterns will be obtained on agarose gels in subsequent electrophoretic analysis. Analysis using capillary electrophoresis is possible, too.

PCR-RLFP is a frequently used method for genotyping of bacteria because of its feasibility, capacity and low costs. Different PCR products are characterized by different restriction patterns that are dependent on the used restriction enzymes. Several enzymes were used for *fla*A typing such as *Hinf*I (Owen et al., 1994), *Alu*I (Burnens et al., 1995) or *Dde*I (Nachamkin et al., 1996). The restriction enzyme *Dde*I was used in many studies demonstrating its efficiency (Nachamkin et al., 1993; Nachamkin et al., 1996; Harrington et al., 1997; Petersen and On, 2000; Fitzgerald et al., 2001, Nguyen et al., 2016). In the fact, the *fla*A typing is considered as a simple, effective and cheap method to investigate the genetic diversity among *Campylobacter* isolates which were collected from various sources or areas (Nguyen et al., 2016).

PCR-RFLP was also used to detect point mutations associated with macrolide resistance (erythromycin) in *Campylobacter* spp. (El-Adawy et al., 2012; Nguyen et al., 2016). The disadvantage of this method is sometimes a low discriminatory power compared with DNA fingerprinting analysis by amplified fragment length polymorphism or pulsed-field gel electrophoresis (Fitzgerald et al., 2001).

1.6.2. Multilocus sequence typing

Multilocus sequence typing (MLST) is a novel approach for molecular epidemiology which was firstly proposed for characterizing *Neisseria meningitides* and *Streptococcus pneumoniae,* it became available in 1998 (Enright and Spratt, 1999). MLST is based on DNA sequences of several housekeeping genes. For seven housekeeping genes, the different sequences are assigned to alleles. These alleles give profiles, which are used to define sequence type (ST) and clonal complexes (CC) of an isolate. Approximately 450-500 bp of an internal fragment of each gene are sequenced. Sequence data are analysed online (http://pubmlst.org) to determine ST and CC.

MLST has demonstrated its effectiveness for discrimination of *C. jejuni* isolates from a wide range of sources and geographical locations (Dingle et al., 2001; Suerbaum et al., 2001; Nguyen et al., 2016). The following gene loci were used for *C. jejuni*: *asp*A (aspartase A), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyl transferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *unc*A (ATP synthase alpha subunit). Eight hundred and fourteen isolates of *C. jejuni* were characterized by Dingle et al. (2002). The study showed a high diversity of isolates. Seven hundred and fourty eight isolates (92%) belonged to one of 17 clonal complexes; most of the human isolates were found in six of

them. Another study of 194 *C. jejuni* isolates of diverse origins, from humans, animals, and the environment showed 155 STs (Dingle et al., 2001). This observation indicated intra- and interspecies horizontal genetic exchange. Nguyen et al. (2016) found 7 sequence types from 9 isolates collected from poultry and pigs in Vietnam. The major advantage of MLST is the comparability of results independent from the laboratory and the local working conditions (technicians, machines etc.). However, the relatively high costs of this complex technique are outweighed by the hard facts that are obtained by sequencing of seven house-keeping genes.

1.6.3. Microarray analysis

DNA microarray analysis is a modern technique based on hybridization of labelled PCR products with a number of specific nucleic acid targets immobilized on a solid surface. This method has been used for three purposes: rapid identification, genotyping and gene expression studies (Ehrenreich, 2006). There are several systems of DNA microarray devices including the ArrayTube™ and ArrayStrip™ systems that are designed by Alere (Jena, Germany) (www.alere-technologies.com).

A DNA microarray assay has been recently developed for the rapid identification of thermophilic *Campylobacter* spp. (Volokhov et al., 2003). *C. coli* and *C. jejuni* are identified using oligonucleotide capture probes which target either the 16S rDNA and 23S rDNA genes, Cj0046 gene locus or the *hip*O gene (Keramas et al., 2003). A recent study of Vietnamese *Campylobacter* isolates by Nguyen et al. (2016) using the microarray assay showed a high genetic diversity of *C. jejuni* isolates. None of the isolates was identical with any other. This result was confirmed by other methods like *fla*A typing which also showed a high heterogeneity of the *C. jejuni* isolates investivated.

The method is fast and the costs of tests are similar to other genotyping procedures (El-Adawy et al., 2012; Nguyen et al., 2016). Thus, it may be used in future studies that focus on the epidemiology, hygiene management, in human and veterinary medicine as a One Health approach.

2. Epidemiology of *Campylobacter* in Vietnam and Kenya

Both, Vietnam and Kenya are developing countries and veterinarians and physicians have limited knowledge about the zoonotic agent *Campylobacter.* Monitoring and control of

Campylobacter have not been applied frequently and were almost neglected. Transmission of *Campylobacter* to humans via contaminated food, faeces, and environmental material needs to be studied in detail.

In Vietnam chicken and pork meat as source of proteins are the favorite food of the population. However, selling food without inspection and hygienic control is common in retail markets. Slaughtering of animals is even carried out right in the markets. Additionally, chicken and pork meat are sold and/or placed together on the market stands. Therefore, this praxis can be assumed that there is a high risk of transmission of *Campylobacter* to consumers.

Poultry farming is economically important in both Vietnam and Kenya and the majority of chickens is kept in small scale farms and backyards. However, overuse of antibiotics in poultry farming has been a problem. A recent survey of antimicrobial usage in chicken production in Vietnam by Carrique-Mas et al. (2015) showed a high level of antimicrobial usage in chicken farms and even probably higher than 6 times of the levels of its usage reported in some European countries. The most common antimicrobial classes included penicillin, quinolones, macrolides, tetracycline and sulphonamides (Carrique-Mas et al, 2015). Overuse of antibiotics in chicken farming has also limited published in Kenya. Mbugua (2011) showed common antibiotic classes has been used in praxis such as tetracycline (64%), sulphonamides (25%), quinolones (8%).

Antibiotic use by farmers in rearing of chicken is common and is considered to be a good way for promoting growth and improving feed efficiency in these developing countries while the antibiotic use in animal farming has been banned in Europe since 2006 (EU report, 2005). Currently, global concerns on the overuse and misuse of antibiotics for animal production or therapy purposes exist. Antibiotic resistance is emerging in bacteria of different animal species and consequently, a potential link to antibiotic-resistant bacteria of humans was supposed (CDC, 2013).

Only few information on the ecology of *Campylobacter* in Vietnam and Kenya is available.

3. Poultry as a natural host for the zoonotic pathogen *Campylobacter*

Campylobacter spp. are commensal organisms commonly found in poultry, cattle, sheep, and swine. It is well known that thermophilic *Campylobacter* are frequently colonize in several avian species including broilers, laying hens, turkeys, and ducks as well as free living birds (Hopkins and Scott, 1983; Deming et al., 1987; Hafez, 2003; Chiller and Holmes, 2004;

WHO, 2011). Infected poultry and contaminated poultry products are considered a one of the major source of *Campylobacter* infection in human population (Hafez, 2003). Reports showed that the prevalence of *Campylobacter* in poultry is high in many developed countries such as the United States, Canada, UK, and EU countries (Williams and Oyarzabal, 2012; Taylor et al., 2013; Canada report, 2014; Schielke et al., 2014). In developing countries, the prevalence is believed to be also high but *Campylobacter* infections are often neglected. The knowledge about campylobacteriosis is limited in these countries.

The epidemiology of *Campylobacter* remains complicated because of its ubiquitous occurrence. Principally, the caecum of chicken is predominant site of organisms in early day of life, particularly *Campylobacter* species (Newell and Fearnley, 2003; Hermans et al., 2011). But after colonisation *Campylobacter* rapidly multiplies to 10^9 cfu/g caecum content (Wassenaar et al., 1993). Faecal shedding is presumably an important factor for the dissemination of *Campylobacter* to most flocks (Newell and Fearnley, 2003). A mathematical model showed that *Campylobacter* could affect 95% of birds in a flock of 20,000 individuals within 1 week after infection of the first bird (Van Gerwe et al., 2009).

The emergence of antimicrobial resistance of *Campylobacter* isolates has increased dramatically, particularly in turkeys (El-Adawy et al., 2012). The emergence of a high resistance rate and multidrug resistance to three or more classes of antimicrobial agents were also observed. The high resistance was sulphamethoxazole/trimethoprim (76.3%), metronidazole (76.3%), ciprofloxacin (69.7%), naladixic acid (67.1%) and tetracycline (55.3%). Multidrug resistance of *Campylobacter* isolates from domestic chicken in Kenya to three or more classes showed 61.3% of isolates (Nguyen et al., 2016). Frequently, resistance to ciprofloxacin, nalidixic acid and tetracycline are identified.

Zoonoses with public health relevance in poultry caused by *Campylobacter* are highly attention (Hafez et al., 2015). Monitoring the progress of antimicrobial resistance of *Campylobacter* in poultry becomes a growing public health issue.

4. Transmission of *Campylobacter*

4.1. Horizontal transmission

Horizontal transmission of *Campylobacter* occurs within animal populations and from animals to humans. Free-living animals but also mass poultry farming systems to another animal are important risk factor for transmission of *Campylobacter* spp. to the environment,

drinking water or milk. *Campylobacter* isolates found in poultry farms, cattle, and pigs are also present in the environment in the vicinity of the farms (Allen et al., 2008; Ellis-Iversen et al., 2009).

Molecular studies by Ridley et al. (2011) showed that *Campylobacter* strains isolated from broiler houses were similar to *Campylobacter* strains from adjacent dairy farms. A later study by Patriachi et al. (2011) also confirmed that bovine faecal *Campylobacter* strains can colonize chickens. Other studies also revealed the presence of identical *C. jejuni* clones in cattle, humans, sheep, dogs and turkeys (Gilpin et al., 2008; Ragimbeau et al., 2008; Hakkinen et al., 2009; Huang et al., 2009). These findings indicate horizontal transmission and a high risk of transmission of *Campylobacter* on multispecies farms.

Some authors found rodents on farms and flies possibly associated with *C. jejuni* transmission (Berndtson et al., 1996; Nichols, 2005; Hald et al., 2008). These animals were supposed to be potential vectors for *C. jejuni*.

Several other vectors were discussed such as untreated water or contaminated (Bull et al., 2006, Messens et al., 2009). Personnel and farm equipment including trucks, forklifts, pallets, crates, footwear have also been found as possible sources of *Campylobacter* infection in poultry (Ramabu et al., 2004).

4.2. Vertical transmission

Vertical transmission of *Campylobacter* is discussed controversially. It remains unclear whether *Campylobacter* can pass from one generation of poultry to the next one via the fertile eggs. An investigation in turkey eggs found no *Campylobacter*, the authors consequently concluded that vertical transmission is a rare event (Acuff et al., 1982; Hafez et al., 2001). No evidence of vertical transmission of *Campylobacter* spp. from grandparent flocks from Sweden to their progeny reared in Iceland could be demonstrated despite the finding that isolates from grandparent birds and parent birds showed identical genetic fingerprints (Callicott et al., 2006). Cox et al. (2012) explained that studies in the 1990s were lacking ideal cultural procedures so that these studies of vertical transmission are of limited use.

In 1985, Clark and Bueschkens (1985) found *Campylobacter* in 11% of 2-day-old hatching chicken eggs. Maruyama and Katsube (1990) demonstrated that *Campylobacter* could be transmitted to the offspring via the hatching eggs after orally infected Japanese quail breeder hens. Through molecular testing, *Campylobacter* has been detected in hatchery fluff, intestinal tracts of developing embryos, and newly hatched chicks (Chuma et al., 1994;

Chuma et al., 1997; Hiett et al., 2002; Hiett et al., 2003). Recently, Hiett et al. (2002) detected *Campylobacter* DNA on eggshell samples and in gastrointestinal contents of unhatched chicken embryos at 7, 15 and 19 days of incubation. Idis et al. (2006) examined the ileal, caecal and yolk contents at the day-of-hatch chicks and they were able to detect *Campylobacter* DNA before the chicks consumed any food or water.

5. Thermophilic *Campylobacter* infection in humans

Poultry (especially chicken) is the major source for *Campylobacter* infection in humans, in particular with *C. jejuni* and *C. coli* (WHO, 2001; Hafez, 2003). Human infections mostly occurs after consumption and handling contaminated food (Berrang et al., 2001; EFSA, 2008; ECDC, 2011). *Campylobacter* contaminated meat could be responsible for up to 40% of human campylobacteriosis cases in European countries (WHO, 2001; ECDC, 2011). In fact, more than 200,000 confirmed cases of human campylobacteriosis were reported by the 24 member states of the EU in 2007 and 190,566 reported confirmed cases in 2008 (EFSA, 2010). In the United States, 21% of culture-confirmed cases of campylobacteriosis are hospitalized. In developing countries, *Campylobacter* infections are under-reported, but are common in diarrhoeal children in an age under five years (5% to 20%) (Coker et al., 2002).

5.1. Health impact of *Campylobacter* infections

Thermophilic *Campylobacter* cause a wide spectrum of diseases including gastroenteritis, proctitis, septicaemia, meningitis, Guillain-Barré syndrome (GBS) (WHO, 2011) whereby in 80% of cases *C. jejuni* and 10% *C. coli* are the causing agents of campylobacteriosis. Clinical presentations of *Campylobacter* infection often range from watery, non-bloody, acute diarrhoea to severe inflammatory diarrhoea with abdominal pain and fever. These symptoms are self-limiting or last for 7 days or more. Infants and young children as well as travelers are the most common groups affected. *Campylobacter* are considered to have an association with GBS and HIV (human immunodeficiency virus) patients (Platts-Mills and Kosek, 2014). *C. jejuni* isolates were found frequently in GBS and HVI patients in China, India and South Africa (Coker et al., 2002). This association of *Campylobacter* with GBS and HIV disease should be studied to further understand more about epidemiology of campylobacteriosis.

5.2. Burdens of *Campylobacter* infections

Campylobacteriosis in humans can be accompanied with following symptoms: diarrhoea, abdominal pain, fever, headache, nausea, and vomiting (Eberle and Kiess, 2012). Costs of approximately $1.2 billion sum up annually (USDA, 2014). Patients with GBS experience a rapid decline in muscle strength and limbs and respiratory system are affected (Nachamkin et al., 1998). In the US, the mean cost per patient with GBS syndrome is around $318.966 and total costs of $1.7 billion annually are expected (Frenzen, 2008). In European countries, *Campylobacter* infections are frequently reported in foodborne illness. EFSA estimated costs of around €2.4 billion a year due to treatments and lost productivity (EFSA, 2012).
In general, economic burdens as well as clinical aspects should be taken seriously.

6. Antimicrobial resistance in *Campylobacter*

Macrolides and fluoroquinolones (FQs) are frequently the drugs of choice for human treatments (Giacomelli et al., 2012). This emerging antibiotic resistance is of global concern.

6.1. Resistance to fluoroquinolones (for example ciprofloxacin)

Fluoroquinolones are antibiotics with quinolone structure for example nalidixic acid. They target two bacterial enzymes which are important for DNA replication, DNA gyrase (type II topoisomerase) and DNA topoisomerase IV (Taylor et al., 2005). They lead to inhibition of synthesis of bacterial DNA causing cell death. Fluoroquinolones are antibiotics with a broad spectrum effect against a wide range of aerobic Gram-positive and Gram-negative bacteria. Ciprofloxacin is one of the fluoroquinolones which is commonly used for treatment of human *Campylobacter* infections. Fluoroquinolone resistance of *Campylobacter* spp. is commonly caused by a mutation of amino acid 86 of the GyrA protein (threonine-to-isoleucine). This mutation appears to be equivalent to the serine-to-alanine mutation of amino acid 83 in the *E. coli* GyrA protein (Ge et al., 2005). However, other sites of mutations have been reported to be associated with fluoroquinolone resistance in *Campylobacter* spp. such as Asp-90-Asn, Thr-86-Lys, Thr-86-Ala, Thr-86-Val and Asp-90-Tyr (Wieczorek and Osek, 2013). Differences between phenotypes and genotypes of fluoroquinolone resistant isolates can be possibly explained by this mechanism (El-Adawy et al., 2012; Nguyen et al., 2016).

Another factors which have been reported to cause fluoroquinolones resistance is the CmeABC multidrug efflux pump (Lin et al., 2002). The efflux pump CmeABC in *Campylobacter* is constructed from three components: an outer membrane protein (encoded

by *cme*C), an inner membrane drug transporter (encoded by *cme*B), and a periplasmic protein (encoded by *cme*A) (Lin et al., 2002). The combination between the CmeABC multidrug efflux pump and *gyr*A mutations resulted in fluoroquinolone resistance (Luo et al., 2003). Luo et al. (2003) reported that values of the minimum inhibitory concentration (MIC) for ciprofloxacin were also reduced in susceptible strains when the efflux pump was blocked. Moreover, the CmeABC efflux pump plays a key role in acquisition of susceptibility to different antibiotics.

6.2. Resistance to tetracyclines

Tetracyclines are a group of broad spectrum antibiotics against both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001) and were used widely in treatment in human and veterinary medicine during the 1950s and 1960s (Speer et al., 1992). Today tetracyclines in human have been used less frequently due to the associated side effects on bone and teeth development, particularly in feces and children.

Tetracylines were divided into atypical and typical tetracylines (Oliva and Chopra, 1992; Chopra, 1994; Chopra and Roberts, 2001). The atypical tetracyclines disrupts bacterial membranes, while typical tetracylines bind the ribosome and inhibit the elongation phase of protein synthesis (Chopra and Roberts, 2001).

In particular, mechanism for tetracycline resistance is the inhibition of protein synthesis which is mediated by a number of *tet* determinants (Manavathu et al., 1990; Connell et al., 2003). The high-level tetracycline resistance of *Campylobacter* is usually associated with the *tet*(O) gene (Pratt and Korolik, 2005). Chopra et al. (2001) described that tetracycline binds to Mg^{2+} that aids to pass through outer membrane porins and then, dislodges from magnesium and moves passively into the cytoplasm to bind to the A site on the ribosomal 30S subunit which results in inhibiting peptide elongations. The presence of the *tet*(O) gene on the chromosome of *C. jejuni* and *C. coli* is responsible for 33-76% of tetracycline resistance as reported for Australia and Canada (Gibreel et al., 2004; Pratt and Korolik, 2005).

6.3. Resistance to macrolides

Macrolides are safe and effective antibiotic agents which are widely used in treatment of bacterial infections. Erythromycin is usually used as a drug of choice to treat cases of human

campylobacteriosis, while other members of this class of antibiotics are approved for veterinary use. Mechanisms of macrolide resistance of *C. jejuni* and *C. coli* have been associated with point mutations in the peptidyl encoding region in domain V of the 23S rRNA gene at positions 2074 and 2075. The mutation at position 2075 is quite common and confers a high-level resistance to macrolide antibiotics (Gibreel and Taylor, 2006; Lin et al., 2007; Ren et al., 2011). Besides, modifications of the ribosomal proteins L4 and L22 may cause resistance to macrolides among *Campylobacter* isolates (Cagliero et al., 2006). The CmeABC efflux pump causes macrolide resistance in *Campylobacter* (Payot et al., 2004; Cagliero et al., 2006).

Resistance to macrolides was observed during 2008 in both *C. jejuni* and *C. coli* in US (2.3% and 10.1%, respectively). A higher resistance was found in EU (10% for *C. jejuni* in Malta and 33% for *C. coli* in Italia) in 2010, and particularly a much higher resistance was seen in Asia and Africa (80% in Nigeria) (Iovine, 2013).

6.4. Resistance to aminoglycosides

Aminoglycosides are antibiotics that inhibit protein synthesis of bacteria. Members of this group include gentamicin, kanamycin, amikacin, neomycin, tobramycin and streptomycin. NARMS reported that more than 99% of *C. jejuni* and 88% of *C. coli* were susceptible to aminoglycosides in 2010 (NARMS, 2010). The main mechanism of aminoglycoside resistance in *C. jejuni* is modification of enzymes which are usually plasmid-borne and the first resistance was found in *C. coli* which was mediated by 3'-aminoglycoside phosphotransferase (encoded by *aph*A-3) (Lambert et al., 1985; Iovine, 2013). The *aph*A-3 gene seems to be the most common source of aminoglycoside resistance in *Campylobacter*. *aph*A-3 has been found in the downstream of an insertion sequence or in the genes encoding streptomycin resistance (Gibreel et al., 2004). It is suggested that *Campylobacter* acquire these genes via horizontal gene transfer.

The vulnerability of efflux pump is still unclear in *Campylobacter* because of missing data. It was observed that the putative efflux pump inhibitors phenyl-arginine-ß-naphthylamine and 1-(1-naphthylmethyl)-piperazin did not affect the MIC of kanamycin in 5 *C. jejuni* isolates (Hannula and Hänninen, 2008). The effect of the putative efflux pump CmeG was also seen in gentamicin resistant *Campylobacter* strains which owned the mutated CmeG, but showed no effect on streptomycin resistance (Jeon et al., 2011).

6.5. Resistance to other antimicrobial agents

Mechanisms of *Campylobacter* resistance to other antibiotic classes have not been investigated extensively, including that ß-lactams AB and sulphonamides (Reina et al., 1994). With the exception of some carbapenems, the majority of *Campylobacter* strains is resistant to penicillins and narrow-spectrum cephalosporins (Martin and Kaye, 2004).

There are three described mechanisms for ß-lactam AB resistance in *Campylobacter*, i.e. enzymatic inactivation by chromosomally encoded beta-lactamases and reduced uptake due to alterations in outer membrane porins and efflux (Iovine, 2013). The cellular death results from binding to penicillin-binding proteins and disrupting peptidoglycan crosslinking during bacterial cell wall formation. Besides, changes in the membrane structure and/or in porin proteins and the efflux pump system can also cause resistance to ß-lactam AB (Martin and Kaye, 2004; Luangtongkum et al., 2009). Due to ß-lactamases *C. jejuni* and *C. coli* isolates are able to inactivate the ß-lactam molecule by hydrolyzing the structural lactam ring. However, amoxicillin and ampicillin resistance has not described yet (Luangtongkum et al, 2009). Susceptibility was demonstrated based on CmeABC-inactivated *C. jejuni* mutants resulting in a significant increase of ampicillin resistance (Lin et al., 2002; Pumbwe et al., 2005).

The mechanism of chloramphenicol resistance is the inhibition of bacterial protein biosynthesis to prevent peptide chain elongation, while sulphonamide resistance is chromosomally mediated through mutational substitution of four amino acid residues in the enzyme dihydropteroate synthetase (Aarestrup and Engberg, 2001; Schlunzen et al., 2001).

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CHAPTER 2

Thermophilic *Campylobacter* - neglected foodborne pathogens in Cambodia, Laos and Vietnam

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Abstract

Thermophilic *Campylobacter* are the most common bacterial cause of gastroenteritis in humans worldwide. Poultry and poultry products are the main sources for human infections. Epidemiological data concerning campylobacteriosis in Asia are limited. Overall, it is difficult to accurately assess the burden of *Campylobacter* infections. South-East Asia including Cambodia, Laos and Vietnam is known as a hotspot for emerging diseases. Campylobacteriosis is a problem of public health concern in these countries, hence. Epidemiological data are scarce. This is influenced by the limited number of laboratory facilities and lack of equipment and awareness in physicians and veterinarians resulting in the lack of surveys.

This review lists articles and reports on *Campylobacter* and campylobacteriosis in these developing third world countries. Subjects are prevalence of thermophilic *Campylobacter* in humans, animals and food and their resistance to several antibiotics.

Keywords: *Campylobacter*, Foodborne zoonoses, Cambodia, Laos, Vietnam, Antibiotic resistance

INTRODUCTION

Zoonoses are diseases and infections which can be transmitted from animals to humans or *vice versa.* Over 200 pathogens are recognized as zoonotic agents and classified as foodborne and non-foodborne agents. Zoonotic foodborne pathogens may cause human diseases after uptake of contaminated food or water. Several of these microorganisms can be found in the intestinal tract of healthy food-producing animals e. g. thermophilic *Campylobacter* species.

Thermophilic *Campylobacter* species are the most common bacterial cause of gastroenteritis in humans worldwide. Incidence and prevalence of campylobacteriosis have increased in both, developed and developing countries, over the last 10 years (Kaakoush et al., 2015). A dramatic increase in the number of reported cases was recognized in Australia, Europe, and North America. In the United States, an incidence of 14.3 campylobacteriosis cases per 100.000 inhabitants was reported for the period between 1996 and 2012 (Gilliss et al., 2012). In Quebec, Canada an annual incidence of 35.2 cases per 100,000 persons was reported (Arsenault et al., 2012). In Europe, *Campylobacter* has become the most frequently reported bacterial pathogen causing gastrointestinal infections in humans since 2005. In 2013, 214,779 confirmed cases were reported by the member states of the European Union (EU) which correlated with a notification rate of 64.8 per 100,000 inhabitants (EFSA, 2013). Hence, the number of fatal cases was very low with 0.05%. Data from African countries are limited and indicate that *Campylobacter* infections are most prevalent in children. In a study in Malawi, 14% of non-diarrheic children and 28% children with diarrhea were PCR positive for *C. jejuni* and *C. coli* (Mason et al., 2013). *C. jejuni* and *C. coli* were also found to be endemic in children in Madagascar and Kenya (Swierczewski et al., 2013; Randremanana et al., 2014). Epidemiological data concerning campylobacteriosis in Asia are limited. A study from China reported that 5% of diarrheic patients were PCR positive for *C. jejuni*. The highest prevalence was detected in the cohort of children younger than 7 years (Huang et al., 2009). Also in

Japan and India *Campylobacter* infections occur quite frequently (Kubota et al., 2011; Mukherjee et al., 2013). Overall, it is difficult to accurately assess the burden of *Campylobacter* infections in Asia owing to insufficient epidemiological data (Kaakoush, 2015).

International travel, consumption of undercooked chicken and products thereof, environmental exposure, and direct contact with farm animals were recognized as risk factors for human Campylobacteriosis (Domingues et al., 2012). The most important sources of foodborne campylobacteriosis are consumption of contaminated food, especially poultry products, unpasteurized milk and water. Broilers are the main source for thermophilic campylobacters to humans (Skirrow, 1977). Studies in Switzerland estimated that 71% of human cases were caused by uptake of contaminated chicken meat (Kittl et al., 2013; Wei et al., 2015). The UK Food Standards Agency found 72.9% of fresh retail chicken *Campylobacter* positive with nearly 20% being highly contaminated (FSA, 2014). Besides broilers, turkeys and ducks, and also cattle and pigs serve as reservoirs of thermophilic *Campylobacter*. *Campylobacter* contaminated water was responsible for outbreaks of human campylobacteriosis, but also for transmission within animal populations (Kaakoush et al., 2015).

South-East Asia is known as a hotspot for emerging diseases. Part of the region is former French Indochina including Cambodia (Kingdom of Cambodia), Laos (Lao People´s Democratic Republic) and Vietnam (Socialist Republic of Vietnam) with a shared history since the 19th century. All three countries are developing third world countries suffering the aftermath of the Vietnam War. Campylobacteriosis is a problem of public health concern in these countries, hence. Epidemiological data are scarce. This may be caused by the limited number of laboratory facilities and lack of equipment and awareness in physicians and veterinarians resulting in the lack of surveys. A recent review of foodborne bacterial and

parasitic zoonosis in Vietnam summarized a number of studies on thermophilic *Campylobacter* infections (Carrique-Mas et al., 2013) but no data on the prevalence of campylobacteriosis are available for Laos and Cambodia.

Therefore, in this review we summarize literature on *Campylobacter* affecting human and animal populations, their prevalence as foodborne pathogen and the resistance to antibiotics in these countries from 1971 to 2016. We delineate knowledge and capability gaps, which will foster new research and surveillance programs. This will help to tackle the impact on public health that is caused by *Campylobacter* infections in the respective countries.

METHODS

Information presented in this review was collected by searching published studies on database including CABDIRECT, Science Direct, Pubmed and Google with keywords "*Campylobacter* and Vietnam", "*Campylobacter* and Laos", "*Campylobacter* and Cambodia". The searched publications were reviewed and relevant information was retrieved. All articles or studies provided information on prevalence of thermophilic *Campylobacter* in humans, animals or food and/or information on antibiotic resistance in these bacteria. All articles and studies were in relation to South-East Asian countries of Cambodia, Laos and/or Vietnam.

Due to limitations of studies, thirty-one publications were retrievable in Pubmed and other database concerning *Campylobacter* in Cambodia, Laos and Vietnam between 1971 and 2016. Nineteen articles were related to Vietnam, 3 articles to Laos and 9 articles to Cambodia.

Eleven articles were related to antibiotic resistance but most of the papers dealt with investigations of the prevalence of *Campylobacter* in humans, animals and food.

RESULTS

Infection of *Campylobacter* in humans

An overview about papers concerning *Campylobacter* in humans in Vietnam, Laos and Cambodia is given in Table 1. The main source of human campylobacteriosis is the consumption of chicken meat, but meat of other species can also be contaminated with *Campylobacter*. Identification of thermophilic *Campylobacter* was carried out by cultivation in combination with biochemical methods. Only recently, identification and differentiation by PCR assays was introduced. Detection rates ranged between 0 and 12% depending on country and the method of detection. *C. jejuni* was detected more often than *C. coli*. Children under 5 years of age are most frequently affected. The risk of infections seems to be correlated with undernutrition, poor hygiene, keeping of animals in the house, manure and wet litter in house yards and contaminated drinking water (Osbjer et al., 2016a). In Cambodia, 12% of 681 human faecal samples were tested positive by PCR assays (Osbjer et al., 2016b). Rates for Vietnam and Laos were below 5%. In an investigation in children with and without diarrhea in Ho Chi Minh City, Vietnam, 2.2% of stool samples were positive, but in the control group without diarrhea 2.6% samples were also found positive (Thompson et al., 2015). A similar result was reported from Cambodia. In Phnom Penh 4.7% *C. jejuni* and 1.5% *C. coli* were detected in diseased children at an age under 5 years and 6.2% *C. jejuni* and 2.4% *C. coli* in the control group (Meng CY et al., 2011). It seems that *Campylobacter* is widespread in the population, but the mere presence of the bacteria in the gut is not inevitable related to clinical symptoms of campylobacteriosis.

In a detailed study from Cambodia, Osbjer et al. (2016b) could not detect *Campylobacter* in 681 stool samples by cultivation of frozen samples. Hence, 66 *C. jejuni* and 16 *C. coli* were identified by multiplex PCR. In the group of children up to 15 years, *Campylobacter* was

detected in 18.8% of the samples whereas only 7% to 8% of those of male and female group over 15 years were *C.* positive. Risk factors for human campylobacteriosis were slaughtering of domestic animals, allowance of animal access to sleeping and food preparation areas and eating of undercooked meat (Osbjer et al., 2016a).

Prevalence of *Campylobacter* in animals and meat

In an investigation from the Mekong delta, Vietnam, the prevalence of *Campylobacter* in faeces of chickens, ducks and pigs was reported to be 31.9%, 23.9%, and 53.7%, respectively (Carrique-Mas et al., 2014). Similar results were found in Cambodia (Osbjer et al., 2016b). In 41.3% of swab and faeces samples of chickens, ducks, pigs and cattle *Campylobacter* was detected by multiplex PCR. 56.1% of chicken and 23.8% of duck samples were positive. 72.2% of pigs but only 5.3% of cattle samples were tested positive for *C. jejuni* and *C. coli*, respectively. *C. jejuni* was the dominant species in chickens and ducks, *C. coli* was more prevalent in pigs. The low prevalence rate of *Campylobacter* in cattle (5.3%) was similar to that in buffaloes in Laos i. e. 2% (Boonmar et al., 2007). A remarkable difference was observed between cultivation and PCR assays. In contrast to 352 samples that were assessed to be positive by PCR assays (41.3%) only 106 samples were identified as *Campylobacter* positive by cultivation (12.4%). Cultivation of *Campylobacter* is difficult at least under field conditions because of their sensitivity to oxygen and changes in temperature.

Contamination rates of poultry products with thermophilic *Campylobacter* were determined to be between 15% and 35% in Vietnam (Table 2). Schwan, 2010 found 76.0% of swabs of chickens positive for *Campylobacter,* but none of the investigated meat samples was contaminated (Schwan, 2010).

In Phnom Penh, Cambodia, was shown that 80.9% of poultry carcasses were contaminated (Lay et al., 2011). The result was obtained by cultivation of *Campylobacter*. A lower contamination rate of 35.0% was reported for poultry products from markets in the capital of Cambodia (Otto, 2012). *C. jejuni* (44.4%), *C. coli* (36.5%), *C. lari* (15.9%) and *C. upsaliensis* (3.2%) were identified among 63 *Campylobacter* isolates.

In a study concerning the prevalence of thermophilic *Campylobacter* in cynomolgus monkeys (*Macaca fascicularis*) kept in captivity and semi-free-range outdoor areas in Japan, these bacteria were detected in 36% of animals of a group imported from Cambodia, but not in animals from Vietnam (Koga et al., 2015).

Table 2 gives an overview about reports on *Campylobacter* in animals and meat in the three Southeast Asian countries.

Antibiotic resistance of *Campylobacter* spp.

Information on antibiotic resistance of thermophilic *Campylobacter* isolates is very limited in Vietnam and Cambodia, and no was published data about antimicrobial susceptibility of *Campylobacter* in Laos were found. Disc diffusion, agar dilution and broth microdilution test were methods for determination of antibiotic resistance of *Campylobacter*. *Campylobacter* isolates were highly resistant to nalidixic acid (58% up to 100%; Table 3) with one exception of 7% (Isenbarger et al., 2002). Resistance to ciprofloxacin was in the range from 7% up to 100% (Table 3). Resistance rates to erythromycin were found between 0% and 100% depending on country, source or method of investigation.

In one report, no difference was found in the prevalence of resistance to several antibiotics between different host species (Carrique-Mas et al., 2014). The resistance profiles were

identical for *C. jejuni* and *C. coli* isolates. Generally, the resistance rate of in *C. coli* isolates is higher than that of *C. jejuni*.

Remarkable was resistance to chloramphenicol with up to 25% in some reports, because use of chloramphenicol is banned in animal breeding in Europe for more than 20 years, but it is still often used in many third world countries (EFSA, 2014). *C. coli* isolates were resistant to ciprofloxacin, nalidixic acid, streptomycin and tetracycline (Nguyen et al., 2016).

Resistance rates of Cambodian *Campylobacter* from chicken to ciprofloxacin reached 90.0% for *C. lari* isolates and was lower for *C. jejuni* and *C. coli* with 60.7% and 52.2%, respectively (Otto, 2012). *C. coli* (30.4%) showed a higher resistance rate to erythromycin in comparison to *C. jejuni* (17.9%). Resistance to tetracycline varied around 50% whereupon *C. coli* showed the highest value (56.5%).

Campylobacter isolated from faeces of monkeys were 100% sensitive to erythromycin and chloramphenicol (Koga et al, 2015). Archawakulathep et al. (2014) gave a good overview of perspectives on antimicrobial resistance in livestock and livestock products in ASEAN countries.

Consequence of finding of fluoroquinolone in imported basa catfish from Vietnam was the stop of sale of 350 tons of seafood in the US by the U. S. Food and Drug Administration (FDA) in 2005 (http://www.campylobacterblog.com/). Motivation was the emerging of resistance to enrofloxacin in *Campylobacter* caused by treatment of chickens and turkeys with this antimicrobial agent in poultry production and the risk for human health.

Molecular biological approach of studies on *Campylobacter*

Recently, five genomes of *Campylobacter jejuni* isolates from Vietnam were sequenced. Some of these isolates had a cluster of genes of the type-6 secretion system (T6SS) which

play roles in pathogen-pathogen and host-pathogen interactions. T6SS is associated with virulence, cell adhesion and cytotoxicity toward erythrocytes. Using the marker gene *hcp* (haemolysin co-regulated protein) the T6SS was detected in more than 70 % of Vietnamese human and chicken isolates (Harrison et al., 2014).

Another study gave a detailed characterization of Vietnamese *Campylobacter* isolates (Nguyen et al., 2016). Investigations concerning genotyping and antimicrobial resistance of *Campylobacter* isolates were carried out using *fla*A typing, MLST and DNA microarray assays. Resistance of *Campylobacter* to several antibiotics was determined phenotypically and by molecular biological methods. A limitation was the low number of isolates.

In a study concerning the regional risks and seasonality in travel-associated campylobacteriosis in East Asia including Cambodia, Laos and Vietnam the risk was estimated to be 386 infections per 100,000 Swedish travelers per year. This is the highest value in the world apart from the Indian subcontinent with 1,253 cases per 100,000 travelers per year (Ekdahl and Andersson, 2004).

In an evaluation study of gastrointestinal pathogens in stool samples from diarrheic patients the usefulness of a multipanel pathogen identification system was shown. It represented a sensitive, specific and easy approach as an alternative to classical detection methods (Duong et al, 2016).

DISCUSSION

Thermophilic *Campylobacter* are well recognized as a common foodborne zoonotic pathogen in developed countries but still be neglected in many developing countries. However, little information about *Campylobacter* was reported in the past in the three South East Asian countries of Cambodia, Laos and Vietnam. Due to its limitation, these countries do not have

national surveillance programs for campylobacteriosis. This review has accessed a large number of studies associated with *Campylobacter* and foodborne pathogens written in English and available in Pubmed and other sources about these countries. Our review of thirty-one articles on *Campylobacter* in these countries from 1971 to 2016 reveals a highly neglected common foodborne-zoonotic pathogen.

Often, investigations were related to human infections especially in children of young age in big cities like Hanoi, Vientiane, Phnom Penh or Ho Chi Minh City. *Campylobacter* isolation rates range from 1.5% to 43.2%, in which *C. jejuni* are dominant than *C. coli.* While thermophilic *Campylobacter* prevalence in animal and meat are from 15.3% to 76%, including *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*. With such prevalence *Campylobacter* should be considered and paid attention for further studies.

Knowledge about the prevalence of *Campylobacter* in humans and poultry and the antibiotic resistance is much better in Vietnam than in Laos or Cambodia. The prevalence of *Campylobacter* isolates is pretty high for nalidixic acid (100%), erythromycin (100%), ciprofloxacin (100%), tetracycline (83%). Antibiotics present in medicated feeds for livestock production are usually in Vietnam and Cambodia. In recent years, Vietnam and other countries in Asia-Pacific region have consumed 48% global veterinary antimicrobial market (Otto et al., 2012). An increasing problem is antibiotic resistance in bacteria like *Campylobacter.* In the EU, antibiotic use of antibiotics as growth promotors in food animals was completely banned several years ago. National monitoring and control programmes for antimicrobial resistance in foodborne pathogens have not been established in ASEAN countries yet (Archawakulathep et al., 2014). Limited data on the amount of antibiotics used in the farming industry exist, because there is no effective control, policy or regulation.

There exist no data about the prevalence of *Campylobacter* in milk or water sources, although *Campylobacter* contamination in both could be a risk for humans. Although the common

habit of consumption of unpasteurized milk in children under 5 year of age underlines the relevance of this potential route of transmission in these countries. However, food safety awareness and concepts are existing (Padungtod et al., 2007). Surveillance and collaborative research within the South East Asian countries can clarify the epidemiology of foodborne infections like campylobacteriosis in humans. It can be also important for control of bacterial contamination in livestock and food of animal origin.

Molecular biological approach to studies of *Campylobacter* recently applied for detection in Cambodia (Poly et al., 2015; Koga et al., 2016; Osbjer et al., 2016a,b) and Vietnam (Carrique-Mas et al., 2014; Anders et al., 2015; Nguyen et al., 2016), not seen in Laos yet. Prerequisite of improvement of food safety and as a consequence of human health is the introduction of modern diagnostics. PCR assays are rapid, reliable and comparably cheap, but especially in Laos molecular techniques are practically not in use yet and there is a substantial lack of laboratory infrastructure and equipment in all three countries.

In summary, this is the first review of thermophilic *Campylobacter* in animals and humans in Cambodia, Laos and Vietnam. The findings from past literatures strongly suggest that *C. jejuni and C. coli* are common in both humans and animals. Social-economic factors and lack of disease surveillance and control programmes present a significant risk of pathogen persistence and transmission in human and animal populations. National surveillance programs and international collaborations are needed to elucidate the epidemiology of *Campylobacter* in developing countries such as Cambodia, Laos and Vietnam. As a neglected zoonotic pathogen, close collaboration between veterinary and medical authorities (One Health) on national level is necessary in order to establish an integrated health surveillance and prevention programs.

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Table 1. Studies concerning presence of thermophilic *Campylobacter* in humans

Country	Region	Group	Method	Result	Reference
Vietnam	Red River	1,655 children under 5 years (one year of investigation)	Cultivation	150 *C. jejuni* and *C. coli* (43.2%) from 347 cultures isolated from 2,160 cases of diarrhoea	Isenbarger et al., 2001
Vietnam	Hanoi	83 children under 3 years with persistent diarrhoea		No *Campylobacter*	Ngan et al., 1992
Vietnam	Hanoi	291 children under 5 years with acute diarrhoea (one year of investigation)	Cultivation, enzyme immunoassay	4% *Campylobacter* positive stool samples	Bodhidatta et al., 2007
Vietnam	Ho Chi Minh City, southern Vietnam	1,309 stool samples of children up to 12 months with diarrhoea	Real-time PCR	152 *Campylobacter* positive (11.6%)	Anders et al., 2015
Vietnam	Ho Chi Minh City	1,419 stool samples of children under 5 years with diarrhoea	Cultivation	6 *Campylobacter* positive in 293 norovirus positive samples	My et al., 2013
Vietnam	Da Nang	987 U. S. Marines		No *Campylobacter*	Forman et al., 1971
Vietnam	Ho Chi Minh City	1,419 children with and 609 without diarrhoea over a one- year period	Cultivation	31 *Campylobacter* sp. in stools of diarrheal cases (2.2%) and 16 in samples without diarrhea (2.6%)	Thompson et al., 2015
Vietnam	Hanoi	636 adults observed for 18 months	Cultivation	0.6 % of stool samples *Campylobacter* positive	Trang et al., 2007
Laos	Vientiane	880 patients with diarrhea in an 11 months period	Cultivation	2.4% *C. jejuni* and 2.0% *C. coli*	Yamashiro et al., 1998

Laos	Vientiane	70 patients with diarrhoea in a 13 months period (most of them < 5 years)	Cultivation	2.9% *C. jejuni*	Phetsouvanh et al., 1999
Cambodia	Thai-Cambodian border	65 stool specimen from children younger 2 years	Cultivation, latex agglu-tination test	16% *Campylobacter* positive	Nordlander et al., 1990
Cambodia	Thai-Cambodian border	487 children with diarrhoea under 5 years	Cultivation	107 out of 487 *Campylobacter* positive (22.0%)	Arthur et al., 1992
Cambodia	Phnom Penh	600 children under 5 years with diarrhea and 578 children without diarrhoea	Cultivation	4.7% *C. jejuni* and 1.5% *C. coli* in diseased children; 6.2% *C. jejuni* and 2.4% *C. coli* in control group	Meng et al., 2011
Cambodia	No information	25 *C. jejuni* from children under 5 years	Multiplex PCR	Detection of capsule type	Poly et al., 2015
Cambodia	Villages in 3 provinces	681 stool samples	Cultivation, multiplex PCR	No *C.* detection by cultivation; 12% *Campylobacter* positive in PCR	Osbjer et al., 2016a,b

Table 2. Studies concerning presence of thermophilic *Campylobacter* in animals and meat

Country	Region	Group	Method	Result	Reference
Vietnam	Hanoi	177 samples of raw food (poultry, pork, beef meat, fish, vegetables) from canteens	Cultivation	28.3% of poultry samples were contaminated with *C. jejuni*	Dao and Yen, 2006
Vietnam	Hanoi	100 samples from chicken breast	Cultivation	31.0% were positive for *Campylobacter*	Huong et al., 2006
Vietnam	Ho Chi Minh City	319 broiler carcasses	Cultivation	35.1% were positive for *Campylobacter*	Bao et al., 2006
Vietnam	Mekong delta	96 samples of chicken meat and 96 cloacal swabs from 20 farms	Cultivation, PCR	No *Campylobacter* from meat; 76.0 % of swab samples were *Campylobacter* positive	Schwan, 2010
Vietnam	Ho Chi Minh City	150 chicken nceck-skins	Cultivation	15.3% *Campylobacter* positive	Garin et al., 2012
Vietnam	Mekong delta	634 faecal samples from pigs, chickens, ducks	Cultivation, PCR	Animal level prevalence of *Campylobacter* was 31.9%, 23.9% and 53.7% for chickens, ducks and pigs	Carrique-Mas et al., 2014
Vietnam	Hanoi	9 *Campylobacter* isolates from chicken and pork meat	Cultivation, PCR	Genotyping by PCR-based methods	Nguyen et al., 2016
Laos	Vientiane	82 caecum samples from cattle; 184 caecum samples and 100 bile samples from buffaloes	Cultivation	4 *Campylobacter* isolates from buffaloes	Boonmar et al., 2007
Cambodia	Phnom	152 poultry carcasses	Cultivation	123 carcasses were	Lay et al.,

	Penh			positive for *Campylobacter*	2011
Cambodia	Phnom Penh and peri-urban areas	180 samples from markets	Cultivation	63 samples (35.0%) positive for *Campylobacter* (28 *C. jejuni,* 23 *C. coli*, 10 *C. lari*, 2 *C. upsaliensis*)	Otto, 2012
Cambodia	Kampong Thom [a)]	36 monkeys (*Macaca fascicularis*)	Cultivation, PCR	36.1% were *Campylobacter* positive	Koga et al., 2015
Cambodia	Villages in 3 provinces	753 livestock samples	Cultivation, PCR	342 samples tested positive for *Campylobacter* (42.5%)	Osbjer et al., 2016a
Cambodia	Villages in 3 provinces	853 livestock samples (cloacal swabs and faeces from chickens, ducks, pigs and cattle)	Cultivation, MALDI-TOF-MS, PCR	*Campylobacter* detected in 106 samples by cultivation and in 352 samples by PCR (41.3%)	Osbjer et al., 2016b

*) kept in Japan

Table 3. Studies concerning antibiotic resistance of thermophilic *Campylobacter* of different origin

Country	Region	Source	Number of isolates Method of investigation	Resistance rate to	Reference
Vietnam	-	Human	88 isolates; MIC agar-plate dilution test	NA: 7% CIP: 7% AZM: 0%	Isenbarger et al., 2002
Vietnam	Hanoi	Human		CIP: 27%	Bodhidatta et al., 2007
Vietnam	Mekong delta	Chicken	22 *C. jejuni* and 6 *C. coli*; Broth microdilution test	NA: 64% [b]; 100% [c] ERY: 0% [b]; 33% [c] CIP: 64% [b]; 100% [c] GEN: 9% [b]; 33% [c] STR: 14% [b]; 50% [c] TET: 68% [b]; 83% [c]	Schwan, 2010
Vietnam	Ho Chi Minh City	Chicken	20 chicken neck-skin samples; Disc diffusion test	AMP: 40% ERY: 25% NA: 95% CIP: 95%	Garin et al., 2012
Vietnam	Mekong delta	Chicken, ducks, pigs	202 *Campylobacter* isolates (*C. jejuni* and *C. coli*); Disc diffusion method	ERY: 100% SXT: 99% NA: 92% CIP: 20.8% CHL: < 10%	Carrique-Mas et al., 2014
Vietnam	Ho Chi Minh City	Human	66 *Campylobacter* isolates from children with diarrhea; 16 isolates from non-diarrheal control; E-test using disc diffusion	AMP: 26.3% CIP: 80.0% CHL: 1.5% NA: 84.8% ERY: 7.8% CIP: 68.7% [a] NA: 62.5% [a] CHL: 18.7% [a]	Thompson et al., 2015
Vietnam	Hanoi	Chicken and pig meat	9 *Campylobacter* isolates (8 *C. jejuni* and 1 *C. coli*); Broth microdilution test	CIP: 62.5% [b] NA: 87.5% [b] STR: 62.5% [b] TET: 75.0% [b] CHL: 25.0% [b] ERY: 25.0% [b] GEN: 25.0% [b]	Nguyen et al., 2016

Cambodia	Phnom Penh	Poultry carcasses	139 *Campylobacter* isolates (*C. jejuni, C. coli, C. lari*); Disc diffusion method	CIP: 25.9% ERY: 4.3% GEN: 1.4% NA: 58.3%	Lay et al., 2011
Cambodia	Phnom Penh	Human	23 *C. coli* and 64 *C. jejuni* isolates; Disc diffusion method	NA: 34% [b]; 57% [c] ERY: 2% [b]; 9% [c] CIP: 31% [b]; 57% [c] AMP: 14% [b]; 22% [c] GEN: 0% [b]; 17% [c] SXT: 75% [b]; 87% [c] TET: 27% [b]; 44% [c]	Meng et al., 2011
Cambodia	Phnom Penh and peri-urban areas	Poultry, carcasses, environment	63 *Campylobacter* isolates (*C. jejuni; C. coli; C. lari, C. upsaliensis*) from markets; Agar dilution method	CIP: 61.9% ERY: 22.2% TET: 50.8%	Otto, 2012
Cambodia	Kampong Thom [d]	Cynomolgus monkeys	15 *Campylobacter* isolates; Agar dilution method	CIP: 100% [b]; 100%[c] STR: 0% [b]; 44% [c] GEN: 0% [b]; 44% [c] TET: 13% [b]; 78% [c]	Koga et al., 2015

NA – nalidixic acid; CIP - ciprofloxacin; AZM – azithromycin; AMP – ampicillin; ERY – erythromycin; SXT – sulfamethoxazole-trimethoprim; CHL – chloramphenicol; STR – streptomycin; TET – tetracycline; GEN - gentamicin

[a] control group; [b] *C. jejuni*; [c] *C. coli*; [d] kept in Japan

CHAPTER 3

Genotyping and antibiotic resistance of thermophilic *Campylobacter* isolated from chicken and pig meat in Vietnam

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Genotyping and antibiotic resistance of thermophilic *Campylobacter* isolated from chicken and pig meat in Vietnam

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Abstract

Background: *Campylobacter* species are recognized as the most common cause of foodborne bacterial gastroenteritis in humans. In this study nine *Campylobacter* strains isolated from chicken meat and pork in Hanoi, Vietnam, were characterized using molecular methods and tested for antibiotic resistance.

Results: The nine isolates (eight *C. jejuni* and one *C. coli*) were identified by multiplex PCR, and tested for the presence or absence of 29 gene loci associated with virulence, lipooligosaccharide (LOS) biosynthesis and further functions. *fla*A typing, multilocus sequence typing and microarray assay investigation showed a high degree of genetic diversity among these isolates. In all isolates motility genes (*fla*A, *fla*B, *flh*A, *fli*M), colonization associated genes (*cad*F, *doc*B), toxin production genes (*cdt*A, *cdt*B, *sec*D, *sec*F), and the LOS biosynthesis gene *pgl*B were detected. Eight gene loci (*fli*Y, *vir*B11, Cje1278, Cj1434c, Cj1138, Cj1438c, Cj1440c, Cj1136) could not be detected by PCR. A differing presence of the gene loci *cia*B (22.2 %), Cje1280 (77.8 %), *doc*C (66.7 %), and *cgt*B (55.6 %) was found. *iam*A, *cdt*C, and the type 6 secretion system were present in all *C. jejuni* isolates but not in *C. coli*. *fla*A typing resulted in five different genotypes within *C. jejuni*, MLST classified the isolates into seven sequence types (ST-5155, ST-6736, ST-2837, ST-4395, ST-5799, ST-4099 and ST-860). The microarray assay analysis showed a high genetic diversity within Vietnamese *Campylobacter* isolates which resulted in eight different types for *C. jejuni*. Antibiotic susceptibility profiles showed that all isolates were sensitive to gentamicin and most isolates (88.8 %) were sensitive to chloramphenicol, erythromycin and streptomycin. Resistance rates to nalidixic acid, tetracycline and ciprofloxacin were 88.9, 77.8 and 66.7 %, respectively.

Conclusions: To the best of our knowledge, this study is the first report that shows high genetic diversity and remarkable antibiotic resistance of *Campylobacter* strains isolated from meat in Vietnam which can be considered of high public health significance. These preliminary data show that large scale screenings are justified to assess the relevance of *Campylobacter* infections on human health in Vietnam.

Keywords: *Campylobacter*, Meat, MLST, Microarray, Antibiotic resistance

Background

Thermophilic campylobacters are the most common bacterial cause of diarrhoea in humans worldwide [1]. Enteric diseases caused by the thermophilic species *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* range from asymptomatic infections to severe inflammatory bloody diarrhoea [2]. *C. jejuni* is often associated with the Guillain–Barré syndrome [3]. Virulence mechanisms in campylobacteriosis are currently poorly understood.

Poultry and poultry products remain the most common source of foodborne human campylobacteriosis [4, 5]. The natural habitat of thermophilic *Campylobacter* is the intestinal tract of healthy birds and raw meat that can be contaminated during the slaughtering process. Consumption of undercooked chicken meat or contaminated ready-to-eat food is the most common source of

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infection. *Campylobacter* are also found in pigs and cattle. Swine carcasses are often contaminated with faeces at the slaughter and processing facilities during the evisceration process, which ultimately leads to contaminated food products [6–8]. Compared to poultry, the relevance of swine in foodborne campylobacteriosis is not well studied. However, a high incidence of *Campylobacter* on pork products at the retail level was found [9, 10].

South East Asia including Vietnam was often considered a hotspot for emerging infectious diseases [11]. Vietnam is currently a developing country and knowledge about *Campylobacter* and campylobacteriosis is limited. Only few data exist about the prevalence of *Campylobacter* in children [12, 13] and adults [14]. The prevalence rates of *Campylobacter* in cases of diarrhea were between 2 and 4 % for children and <1 % for adults. In a study concerning the incidence of diarrhea in rural Vietnamese children [15] *Campylobacter* was the most frequently identified pathogen comprising 31 % of all isolates.

Fifteen to 32 % of meat samples in different regions of Vietnam contained thermophilic *Campylobacter* [16–21]. Duck meat and pork were also contaminated with *Campylobacter* in 23.9 and 53.7 % of tested samples, respectively [21]. Bao et al. isolated thermophilic campylobacters from 35.1 % of chicken carcasses in large and small abattoirs of Ho Chi Minh City and 67.9 % of the isolates belonged to the species *C. jejuni* [22]. However, Schwan investigated meat samples from markets in the Can Tho Province but found no *Campylobacter* spp. [19].

Several molecular biological methods for characterization and discrimination of *Campylobacter* isolates have been developed [23]. PCR and *fla*A typing were used as well as multi-locus sequence typing (MLST) and microarray assays for determination of relatedness among isolates [24, 25].

The molecular genetics of *Campylobacter* has been extensively studied but the pathogenesis of *Campylobacter* infections is not fully understood. A number of putative virulence and toxin genes that may contribute to pathogenicity in human *Campylobacter* infection have partly been identified and sequenced [26–29].

Flagella-mediated motility, adherence to intestinal epithelial cells, invasion and survival in the host cells as well as the ability to produce toxins are important virulence factors [27]. The involvement of the *fla*A gene in *Campylobacter* colonization has been shown [30]. Several *Campylobacter* cytotoxins have been identified [31] and the cytolethal distending toxin (CDT) has been characterized in detail [32, 33]. CDT is composed of three subunits and it has been suggested that CDT, amongst other functions, may play a role in adhesion and invasion [34]. Active CDT is lethal for host enterocytes [35, 36].

It was shown that 19–53 % of *Campylobacter* spp. strains contain plasmids of various sizes [37]. The plasmid-encoded *vir*B11 gene is a marker potentially associated with the virulence of *Campylobacter* species [38].

A study with Vietnamese isolates dealt with the identification of possible virulence markers like a novel protein translocation system, the type-6 secretion system [39].

The antimicrobial resistance of *Campylobacter* isolates was investigated also in several studies [19, 20, 40]. High resistance rates in *C. jejuni* were determined against ciprofloxacin, nalidixic acid and tetracycline with 64, 46 and 68 %, respectively. Resistance against antibiotics in *C. coli* isolates was higher than in *C. jejuni*. All *C. jejuni* isolates were resistant to ciprofloxacin and nalidixic acid while, 83 % showed resistance to tetracycline [19]. These isolates were recovered from faeces. The broth microdilution method is an easy and reliable method for interpreting minimum inhibitory concentration (MIC) values for *C. jejuni* and *C. coli* which is also recommended by EUCAST [41–43]. The emergence of antimicrobial resistance in *Campylobacter*, particularly to fluoroquinolones, has showed the need for continued monitoring of *Campylobacter* resistance.

In this study, Vietnamese *Campylobacter* isolates were characterized to assess their genetic relatedness, potential virulence factors and antibiotic resistance profiles. The isolates were recovered from chicken and pig meat from two slaughterhouses in Hanoi. The investigation was done using different molecular biological tests, MLST, microarray analysis, and the antimicrobial susceptibility was assessed.

Methods

Campylobacter isolates

Campylobacter were isolated from 100 chicken meat and 50 pork samples of two slaughterhouses in Hanoi, Vietnam, following the International Standards Organization [ISO] 10272-1 (2006) guidelines [44] by the Institute of Veterinary Science in Hanoi, Vietnam, in 2009. Bacteria were stored using the Cryobank system (Mast Diagnostica, Reinfeld, Germany) and transferred to the National Reference Laboratory of Campylobacteriosis at the Institute of Bacterial Infections and Zoonoses of the Friedrich-Loeffler-Institut in Jena, Germany. *Campylobacter* isolates were sub-cultured on Mueller-Hinton Agar (Oxoid GmbH, Wesel, Germany) supplemented with 10 % bovine blood under microaerophilic conditions (5 % O_2, 10 % CO_2 and 85 % N_2) at 42 °C for 48 h. Isolates were kept in cryovials at −80 °C.

DNA extraction

Genomic DNA was extracted from 48-h bacterial cultures on Mueller-Hinton blood agar plates using the High

Pure PCR Template Preparation Kit™ according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Extracted DNA was quantified spectrophotometrically using a Nanodrop® ND-1000 (Fisher Scientific GmbH, Schwerte, Germany). DNA extracts were stored at −20 °C.

Species confirmation

Bacterial isolates were identified using a multiplex PCR assay [25] targeting the *map*A and *ceu*E genes.

*fla*A-RFLP typing

*fla*A-RFLP (flagellin A-restriction fragment length polymorphism) typing was performed as described previously [24]. Briefly, a part of the *fla*A gene of the isolates was amplified using primer pair flaA1-Wob/fla2-Wob (Jena Bioscience GmbH, Jena, Germany). The approximately 1700 bp amplicons were digested with *Dde*I (Roche Diagnostics GmbH) as recommended by the manufacturer. The DNA segments were analyzed after electrophoresis on a 1.5 % agarose gel by staining with ethidium bromide and visualization under UV light. Documentation was carried out using a Bio Imaging System (Syngene, Cambridge, UK).

Multilocus sequence typing

Seven housekeeping gene loci including *asp*A (aspartase A), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxyl methyl transferase), *pgm* (phosphor glucomutase), *tkt* (transketolase), and *unc*A (ATP synthase α subunit) were amplified by PCR as described previously [3]. PCR conditions were modified: after initial denaturation at 96 °C for 60 s followed 35 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. Amplicons were examined by gel electrophoresis on a 1.5 % agarose gel and purified with the QIAamp Gel Extraction Kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. Cycle sequencing was carried out using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). Analysis of sequencing products was done with a genetic analyzer ABI PRISM 3130 (Applied Biosystems).

Alleles, sequence types (STs), and clonal complexes (CCs) were assigned by submitting DNA sequences of amplicons to the MLST database available at the following website: http://pubmlst.org/campylobacter.

Microarray DNA hybridization assay

DNA microarray analysis described here was based on the presence or absence of gene loci of *Campylobacter jejuni* isolates using the ArrayTube™ platform (Alere Technologies GmbH, Jena, Germany) [45]. Two types of microarrays with spotted probes were used to differentiate *C. jejuni* isolates: *C. jejuni*-1 and Campy-2. Sample processing was done using a commercial kit (Alere Technologies GmbH) according to the manufacturer's instructions (www.alere-technologies.com). Briefly, 1 µg of genomic DNA was amplified and labelled by PCR with random primers and biotin-16-dUTP. Labelled DNA was hybridized to both microarrays for 1 h at 45 °C, washed, and quantified after colorimetric reaction using horseradish peroxidase and TrueBlue substrate. Hybridization signals were measured after 5 min precipitation with an ArrayTube transmission reader ATR-03 (Alere Technologies GmbH). Interpretation of array data was described by El-Adawy et al. [24]. SplitsTree analysis was done using BioNumerics (version 4.6; Applied Maths NV, Sint-Martens-Latem, Belgium).

Molecular biological characterization of *Campylobacter* isolates

Detection of genes which have functions for motility, adhesion, colonization, invasion, toxin production, lipooligosaccharide (LOS) biosynthesis was carried out by PCRs as described previously [29, 46]. The presence of additional gene loci was detected as described in publications cited in Table 1.

Antimicrobial susceptibility testing and MIC determination

The broth micro-dilution test was performed with Sensititre Campylobacter plates EUCAMP 2 (MCS Diagnostics BV, RE Swalmen, The Netherlands). They consist of 96 round-bottom wells which are pre-coated with various concentrations of seven different clinically used antibiotics. The antimicrobial agents and their concentration ranges used in the test are given in Table 2. The susceptibility tests were performed according to CLSI guidelines [41]. Briefly, *Campylobacter* isolates were cultivated on Mueller-Hinton agar (Oxoid GmbH) supplied with 10 % bovine blood under microaerophilic conditions at 37 °C for 48 h. Bacterial colonies were suspended in NaCl solution (0.9 %) for matching turbidity of 0.5 McFarland units (Dr. Lange, CADAS 30 photometer, Berlin, Germany). One-hundred and fifty µl of the suspension were diluted in 10 ml Mueller-Hinton broth (Oxoid GmbH) resulting in a concentration range of 10^6–10^7 colony forming units (cfu)/ml. Each well was dispensed with 100 µl of the suspension. The plates were sealed and incubated at 37 °C for 24 h under microaerophilic conditions. The results were obtained by reading either visually or photometrically (Tecan Deutschland GmbH, Crailsheim, Germany) using computer program easyWIN fitting (version V6.1, 2000). *C. jejuni* DSM 4688 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and

Table 1 Presence of virulence-associated genes, lipooligosaccharide biosynthesis genes and other gene loci in Vietnamese *Campylobacter* isolates

Species	09CS 0040[a] C.j	09CS 0043[a] C.j	09CS 0046[a] C.j	09CS 0047[a] C.j	09CS 0049[a] C.j	09CS 0067[a] C.j	09CS 0066[b] C.j	09CS 0068[b] C.j	09CS 0051[a] C.c	References
Virulence-associated genes										
*fla*A	+	+	+	+	+	+	+	+	+	[29]
*fla*B	+	+	+	+	+	+	+	+	+	[29]
*flh*A	+	+	+	+	+	+	+	+	+	[29]
*fli*M	+	+	+	+	+	+	+	+	+	[29]
*fli*Y	−	−	−	−	−	−	−	−	−	[29]
*cia*B	+	+	+	+	−	+	+	+	−	[29]
*iam*A	+	+	+	+	+	+	+	+	−	[29]
*vir*B11	−	+	−	+	−	−	−	−	−	[29]
*cad*F	+	+	+	+	+	+	+	+	+	[29]
*doc*A	+	+	+	+	+	+	+	+	+	[29]
*doc*B	+	+	+	+	+	+	+	+	+	[29]
*doc*C	−	−	−	−	+	+	+	−	−	[29]
*cdt*A	+	+	+	+	+	+	+	+	+	[29]
*cdt*B	+	+	+	+	+	+	+	+	+	[29]
*cdt*C	+	+	+	+	+	+	+	+	+	[29]
*wla*N	−	−	−	−	−	−	−	−	−	[29]
*cgt*B	−	−	−	+	+	+	+	+	+	[29]
LOS genes										
*pgl*B	+	+	+	+	+	+	+	+	+	[46]
Cje1278	−	−	−	−	−	−	−	−	−	[46]
Cje1280	−	−	−	+	−	+	−	−	−	[46]
Cj1434c	−	−	−	−	−	−	−	−	−	[46]
Cj1138	−	−	−	−	−	−	−	−	−	[46]
Cj1438c	−	−	−	−	−	−	−	−	−	[46]
Cj1440c	−	−	−	−	−	−	−	−	−	[46]
Cj1136	−	−	−	−	−	−	−	−	−	[46]
Secretory genes										
*sec*D	+	+	+	+	+	+	+	+	+	[77]
*sec*F	+	+	+	+	+	+	+	+	+	[77]
Paralogous gene family										
*maf*1	+	+	−	+	+	+	+	+	+	[78]
*maf*4	+	+	+	+	−	+	+	+	+	[78]
Type VI secretion system										
hcp	+	+	+	+	+	+	+	+	−	[39]

C.j, *Campylobacter jejuni*; C.c, *Campylobacter coli*

[a] Isolated from chicken meat

[b] Isolated from pork

C. coli DSM 4689 were included in each batch of broth micro-dilution test for quality control.

Molecular biological detection of resistance determinants

Erythromycin resistance

Point mutations at positions 2074 and 2075 in domain V of the 23S rRNA were confirmed as the most common mechanism for macrolide resistance in *Campylobacter*. The detection of point mutations was done by MAMA-PCR assay as previously described [47].

Ciprofloxacin resistance

A single point mutation (Thr-86-Ile) in the quinolone resistance-determining region (QRDR) of *gyr*A was

Table 2 MICs and resistance rate of Vietnamese *C. jejuni* isolates

	Concentration range (µg/ml)											R (%)
	<0.06	<0.12	0.25	0.5	1	2	4	8	16	32	64	
Chloramphenicol						6				*2*		25.0
Ciprofloxacin	1	2			*1*	*4*						62.5
Erythromycin				6						*2*		25.0
Gentamicin			3	2	1			*1*	*1*			25.0
Nalidixic acid									1	*1*	*6*	87.5
Streptomycin					1	1	1	*3*	*2*			62.5
Tetracycline			1		1					*6*		75.0

Italic values represent number of resistant isolates

defined as source of high-level resistance to fluoroquinolones [48]. The MAMA-PCR was done to detect *gyr*A mutation in *C. jejuni* and *C. coli* isolates as described by [49, 50] with modified PCR cycling conditions.

Tetracycline resistance

tet(O) gene is strongly associated with tetracycline resistance in *C. jejuni*. Primer pair DMT1/DMT2 was chosen to detect this resistance determinant as described previously [51].

PCR conditions were identical with those described by El-Adawy et al. [52].

Results

Campylobacter species identification

In total, 20 isolates suspected to be *Campylobacter* were cultivated (15 from chicken meat and 5 from pork) in Vietnam, saved cryo-conserved and transferred to Germany. However, only 9 isolates could be re-cultivated on Mueller-Hinton agar. Table 1 gives an overview of origin and species of cultivated *Campylobacter* isolates. Eight isolates belonged to *C. jejuni* and one isolate from chicken meat was identified as *C. coli* by multiplex PCR.

*fla*A-RFLP typing

Vietnamese *Campylobacter* isolates were characterized by *fla*A typing using the restriction enzyme *Dde*I (Fig. 1). The restriction profiles of *C. jejuni* yielded five different types.

MLST

Within the eight *C. jejuni* isolates, six different sequence types were identified (Table 3). Sequence type ST 5799 was found in *C. jejuni* isolates recovered from chicken and pork meat. *C. jejuni* sequence types ST 2837 and ST 4395 found in chicken belonged to the clonal complex ST-353. Four sequence types could be assigned to clonal complexes and two others (ST 5155 and ST 6736) were not assignable.

Campylobacter coli isolate 09CS0051 belonged to clonal complex ST-828.

Microarray DNA hybridization assay

The DNA microarray assay showed high significant genetic diversity among 8 *C. jejuni* isolates (Fig. 2). Isolates 09CS0040 and 09CS0046 were closely related and represented the same sequence type in MLST in which no assignment to an existing clonal complex was possible. Otherwise, isolates 09CS0049 and 09CS0066 belonged to the same sequence type (ST 5299) and CC (ST-443) proved to be different when considerably more gene loci were analyzed using the microarray. Likewise, isolates 09CS0043 and 09CS0047 showed large disparity in microarray analysis independent from their affiliation to ST-353 complex, whereby the different sequence types had to be considered.

Molecular biological detection of different virulence-associated and toxin genes

*fla*A, *fla*B, *flh*A and *fli*M as genes of the flagellar system of *Campylobacter* were found in all isolates, but *fli*Y could not be detected by PCR (Table 1). The invasion-associated gene *iam*A was present in all *C. jejuni* isolates whereas *cia*B was absent in one *C. jejuni* isolate. The gene *vir*B11 was found in two isolates. All *C. jejuni* isolates carried *cad*F (an outer-membrane protein gene), *cdt*A, *cdt*B and *cdt*C-C (cytolethal distending toxin), *doc*A (encoding a periplasmic cytochrome C peroxidase), and *doc*B (encoding a methyl-accepting chemotaxis protein). Detection of *doc*C (another methyl-accepting chemotaxis protein) was variable among the isolates (Table 1). It was detected in three out of eight isolates. *wla*N (a beta-1,3 galactosyltransferase) which is responsible for a specific LOS structure was not identified in any of the isolates. In contrast, *cgt*B (another beta-1,3 galactosyltransferase gene) was found in five of eight *C. jejuni* isolates. The *C. coli* isolate 09CS0051 showed a difference to the *C. jejuni* strains. The invasion-associated genes *cia*B and *iam*A were not

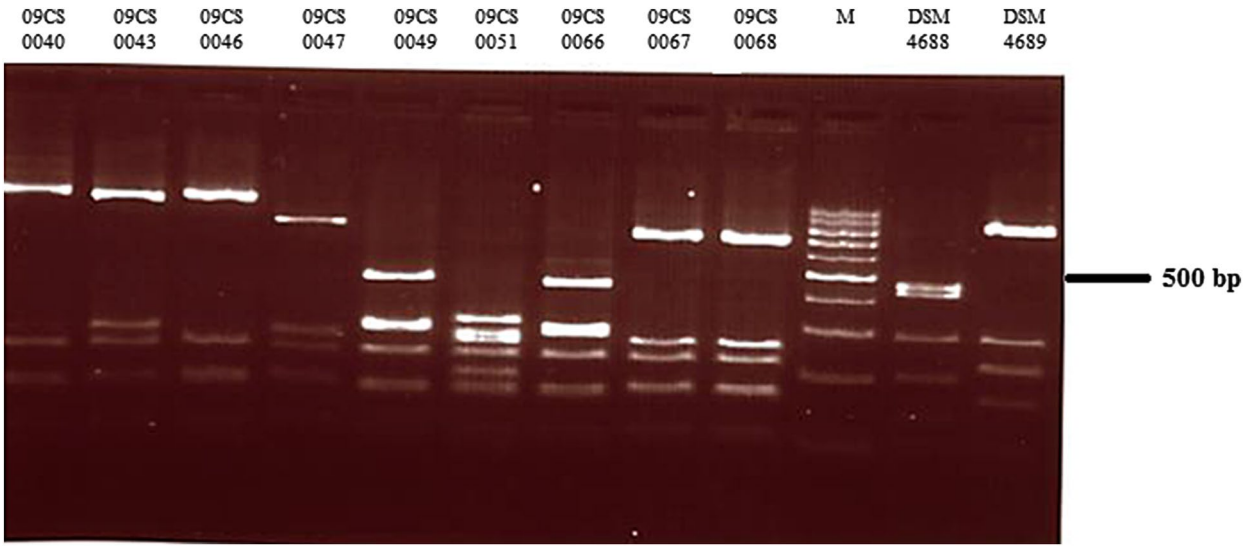

Fig. 1 Agarose gel electrophoresis of *fla*A typing products of Vietnamese *Campylobacter* isolates digested with *Dde*I (M—100 bp DNA ladder)

Table 3 Results of MLST of Vietnamese *Campylobacter* isolates

Isolate	*asp*A	*gln*A	*glt*A	*gly*A	*pgm*	*tkt*	*unc*A	ST	CC	Source[a]	Year[a]	Location[a]
09CS0040	1	2	2	212	11	253	147	5155	–	–	–	–
09CS0043	7	2	5	289	10	3	6	2837	ST-353 complex	–	–	–
09CS0046	1	2	2	212	11	253	147	5155	–	–	–	–
09CS0047	24	17	1	2	11	3	6	4395	ST-353 complex	Human	2010	Vietnam
09CS0049	7	2	2	15	23	3	12	5799	ST-443 complex	Human	2010	Japan
09CS0066	7	2	2	15	23	3	12	5799	ST-443 complex	Human	2010	Japan
09CS0067	9	30	2	2	11	59	6	4099	ST-460 complex	Human	2009	Canada
09CS0068	73	2	250	10	10	59	291	6736	–	Human	2013	Thailand
09CS0051	33	39	30	79	113	47	17	860	ST-828 complex	Human, Poultry, Food	1999–2014	USA, Europe, not Asia

[a] Equivalent types from the MLST database

detected by PCR. The presence of LOS biosynthesis genes was determined to characterize the Vietnamese campylobacters. In all *Campylobacter* isolates *pgl*B (encoding a putative oligosaccharyl transferase) was detected. Gene loci for putative galactosyltransferases (Cje1278, Cje1280, Cj1136, Cj1138, Cj1434c, Cj1438c, Cj1440c) were rarely found. Only Cje1280 was detected in two *C. jejuni* isolates.

Secretory genes *sec*D and *sec*F were detected in all *Campylobacter* isolates. Type-6 secretion system (T6SS), a novel class of protein translocation system, was identified over the haemolysin co-regulated protein (*hsp*) gene in all *C. jejuni* isolates.

The motility accessory factor (*maf*) family represents a new class of bacterial genes related to flagellar biosynthesis and phase variation. *maf*1 and *maf*4 were found in almost all isolates with the exceptions of 09CS0047 which lacked *maf*1 and 09CS0049 where *maf*4 could not be identified by PCR.

Antimicrobial susceptibility testing

The results of antimicrobial susceptibility testing for seven antibiotic agents are given in Table 4. None of the *Campylobacter* isolates was fully susceptible to all investigated antibiotics. The *C. jejuni* isolates were highly resistant to ciprofloxacin, nalidixic acid, streptomycin and tetracycline with 62.5, 87.5, 62.5 and 75.0 % resistance, respectively. The resistance rate for chloramphenicol, erythromycin and streptomycin was low with 25.0 %. *C. coli* isolate 09CS0051 was resistant to ciprofloxacin, nalidixic acid, streptomycin and tetracycline.

Molecular biological assays for detection of ciprofloxacin, erythromycin, and tetracycline resistance

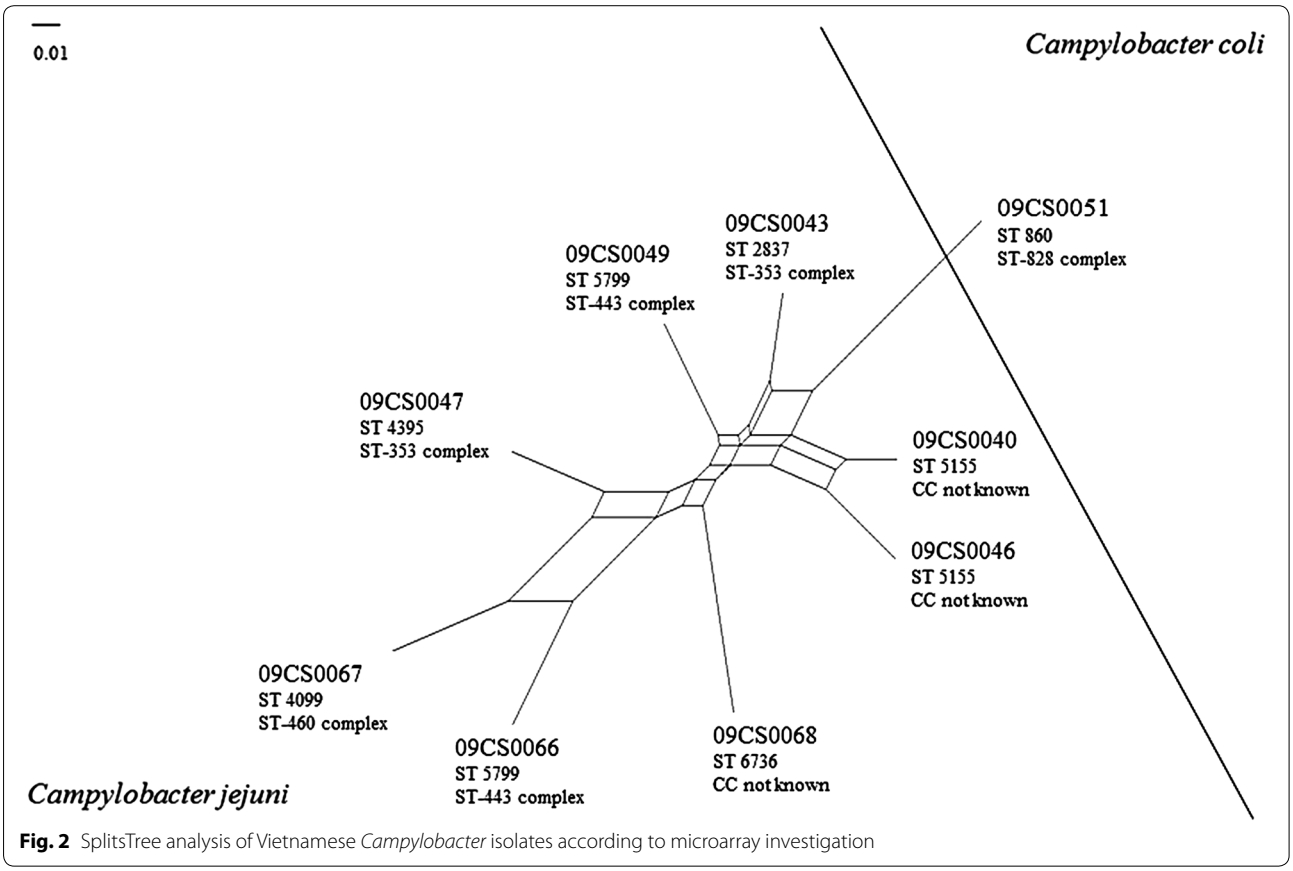

Fig. 2 SplitsTree analysis of Vietnamese *Campylobacter* isolates according to microarray investigation

Table 4 Resistance of Vietnamese *Campylobacter* isolates to various substances

Isolate	Agent						
	CHL	CIP	ERY	GEN	NAL	STR	TET
09CS0040	S	S	S	S	R	S	S
09CS0043	R	R	R	AMB	R	S	R
09CS0046	S	S	S	S	S	AMB	S
09CS0047	S	R	S	S	R	AMB	R
09CS0049	S	R	S	S	R	S	R
09CS0066	S	R	S	S	R	R	R
09CS0067	R	R	R	R	R	R	R
09CS0068	S	S	S	S	R	R	R
09CS0051	S	R	S	S	R	R	R

CHL chloramphenicol, *CIP* ciprofloxacin, *ERY* erythromycin, *GEN* gentamicin, *NAL* nalidixic acid, *STR* streptomycin, *TET* tetracycline, *R* resistant, *S* sensitive, *AMB* ambiguous

determinants confirmed the results of phenotypic testing of antimicrobial resistance.

Discussion

As in other countries, thermophilic *Campylobacter* are common bacterial agents in Vietnam which cause gastro-enteric illness in humans, especially in children [13, 15]. Meat and meat products serve as the main sources of human infections. Huong et al. showed that approximately 30 % of raw chicken samples from school and hospital canteens and retail markets in Hanoi city were contaminated with *Campylobacter* [17]. Other studies from different regions of Vietnam came to comparable results [20, 21]. Here, chicken and pork meat samples

from a market in Hanoi were investigated for the presence of thermophilic campylobacters. 13.3 % of the samples were *Campylobacter* positive. *C. jejuni* and *C. coli* were detected as contaminants of chicken and pork meat. In comparison, a report from Germany showed that 52.3 % of chicken carcasses in slaughterhouses and nearly 40 % of raw meat in retail were positive for these microorganisms [53]. It is possible that the lower percentage of *Campylobacter* findings in the meat samples from the Hanoi market is a result of slaughter procedures. In Vietnam the meat is not prepared in large slaughterhouses for retail as in Germany. Chicken and also pigs are slaughtered in low numbers and often directly on-site and the risk of contamination for example by generation of aerosols in slaughterhouses is smaller.

In this study, 20 *Campylobacter* isolates were obtained from meat samples in Vietnam. Unfortunately, after transfer to Germany only 9 isolates could be re-cultivated which were subject to further investigation. Eight of them were identified as *C. jejuni* and one as *C. coli* by mPCR. Several methods were used to type the isolates. As a rapid and simple method to illustrate heterogeneity within the *C. jejuni* isolates, *fla*A-RFLP typing was used. Four different strain types were detected by *Dde*I digestion of the amplified *fla*A gene. This enzyme was used because it showed the highest discriminatory power in former investigations [24] in comparison with *Alu*I or *Sau*3AI. The digestion pattern of *C. coli* isolate 09CS0051 was completely different. Limitations of this typing method resulted from the use of only a very small part of the *Campylobacter* genome and difficulties in standardization of the analytical process. This complicates an inter-laboratory comparison of results between different laboratories.

Microarray analysis worked as a PCR-based comparative genomic fingerprinting (CGF) assay [54] and confirmed the heterogeneity of the *C. jejuni* isolates. An advantage of this method is the use of the whole genome data instead of only one or a few genes. The basis of this method is the detection of the presence or absence of several gene loci that are spread over the whole genome. SplitsTree analysis of the hybridization results showed high genetic diversity as no isolate is identical with another one. The *C. coli* isolate 09CS0051 was clearly distinct from the *C. jejuni* isolates. Additionally, sequence types and clonal complexes of the isolates determined by MLST are given in Fig. 2.

Both methods showed differences concerning the relatedness of different *C. jejuni* isolates among each other. Isolates 09CS0049 and 09CS0066 represented an identical sequence type and belonged to the same clonal complex but in microarray investigation they showed only poor relatedness. 09CS0043 and 09CS0047 were part of the clonal complex ST-353 but differed in the sequence type. Genetic relatedness based on microarray data was marginal. In contrast, two isolates (09CS0040 and 09CS0046) were found with identical sequence type and pattern in *fla*A typing after *Dde*I digestion and even microarray analysis showed a high degree of similarity.

The major advantage of MLST is the comparability of results independent from the laboratory and the local working conditions (technicians, machines etc.). The relatively high costs of this complex technique are outweighed by the hard facts that are obtained in the form of DNA sequences of seven house-keeping genes. In this study, six sequence types in the group of *C. jejuni* isolates were detected. These sequence types were compared with the database on the *Campylobacter* MLST Home Page (http://pubmlst.org/campylobacter/). ST 2837 and ST 4395 belonged to clonal complex ST-353 whereby 09CS0047 (ST 4395) was identical with an isolate which was recovered from a stool sample of a hospital inpatient with gastroenteritis in Vietnam in 2010. 09CS0049 and 09CS0066 belonged to CC ST-443. Sequence type 5799 was previously isolated from human stool samples in Japan. Three isolates could not be assigned to any known clonal complex. The sequence type of isolate 09CS0068 had previously been discovered once in a human stool sample in Thailand, two others were not described yet. Isolate 09CS0067 represented sequence type 4099. This type belongs to the ST-460 complex and was previously identified in a human sample in Canada. *C. coli* isolate 09CS0051 belonged to sequence type 860 and ST-828. Identical isolates were found several times during the last two decades in Europe and the USA. Records from Asia are lacking until now. In summary, the investigated Vietnamese isolates in their majority seemed to represent strains typical for the Asian region. A route of infection of *Campylobacter* from meat to humans can be assumed.

The *Campylobacter* isolates were characterized regarding virulence factors associated with adhesion and invasion of host cells. All isolates harboured flagellin genes *fla*A, *fla*B, *flh*A and *fli*M. Similar observations have been reported previously [27, 55]. Molecular genetic approaches with defined mutants showed that *fla*A is essential for colonization [30]. The complex flagellum of *Campylobacter* species is encoded by two tandem-oriented flagellin genes (*flaA* and *flaB*). While the function of the *fla*A gene seems to be fully elucidated, there are many speculations about the function of the *fla*B gene, which may play a role in antigenic variation or influence the motility in various environmental conditions [56]. *fliY*, a gene of flagellar motor switch proteins, could not be detected.

The *cia*B gene, coding for a *Campylobacter* invasion antigen, was present in most of the *C. jejuni* isolates. It

was absent in 09CS0049 and *C. coli* 09CS0051. Another gene which is important in the invasion process of *Campylobacter* to host cells is *iam*A. It was detected in all *C. jejuni* isolates. Carvalho et al. described the detection of the *iam*A gene in 85 % of invasive *C. jejuni* but in non-invasive isolates it is rare [57]. Also the *cad*F gene was detected in all *C. jejuni* isolates. It encodes for an outer-membrane protein which mediates the binding of the bacteria to fibronectin [58]. Based on the results it can be concluded that these Vietnamese isolates represented invasive *C. jejuni* strains.

Cytolethal distending toxin causes direct DNA damage leading to induction of DNA damage checkpoint pathways [35]. The *cdt* gene cluster consists of 3 genes *cdt*A, *cdt*B and *cdt*C. The *cdt* genes were shown to be conserved among different *Campylobacter* strains [59]. Bang et al. observed that the presence of these genes in isolates from different sources exceeds 90 % [27]. In all Vietnamese *C. jejuni* strains isolated from chicken and pork meat the complete *cdt* gene cluster was observed. Rozynek et al. obtained results for *C. jejuni* strains isolated from children with diarrhea and found that *cdtA*, *cdtB* and *cdtC* were present in 98.4, 97.0 and 98.0 % of all isolates, respectively [60]. However, *cdt*C was not detected in *C. coli* isolate 09CS0051 from chicken meat which was in agreement with a previous study [60]. On the other hand, a similar frequency of *cdt* genes and the *cdt* gene cluster was observed in dog and chicken isolates [55]. In this study all investigated isolates harboured the *cdt*B gene. It is indeed generally accepted that the *cdt*B genes are widespread amongst poultry and cattle as well as in human isolates in Denmark, Japan, Poland, and Belgium [27, 60–62]. However, low percentages of occurrence of *cdt*B have been reported in humans (28 %) and chickens (20 %) in India, which could be due to genetic reasons or variations in the isolates from different geographic areas [63].

Only a minority of *C. jejuni* isolates gave positive PCR results for *vir*B11 encoding a putative component of a type IV secretion system. It is located in the pVir plasmid and could be involved in virulence [38]. The 25.0 % prevalence of the *virB11* gene in *C. jejuni* isolates in this study is higher than 10.3 % in human isolates reported by Bacon et al., but much lower than in pig isolates (35.7 %). Until now, the role of the protein encoded by the *vir*B11 gene in the invasion and colonization process of eukaryotic cells by *Campylobacter* species could not be elucidated.

Macrolides, quinolones and tetracycline are among the common antimicrobials recommended for testing, because they can be of therapeutic relevance in severe cases of infection. High levels of resistance of *Campylobacter* to tetracycline and ciprofloxacin were frequently reported but resistance to erythromycin and gentamicin remained low.

The antimicrobial susceptibility profiles among the Vietnamese isolates were analyzed based on the guidelines of CLSI (2008) [41]. In this study standardization of the protocol for the commercially available broth microdilution test as a method for the determination of the minimum inhibitory concentration (MIC) of antibiotics was done [52]. All isolates were sensitive to gentamicin and most of isolates (88.8 %) were sensitive to chloramphenicol, erythromycin and streptomycin. Similar results were reported in several previous studies [4, 42, 64–68]. In contrast to our findings, a previous study reported high resistance to streptomycin with 60.0 % [64]. The resistance rate to ciprofloxacin was 66.7 % which is in agreement with a previous study showing high resistance [51, 64], but in contrast to another study with only 9.5 % [69]. Resistance to nalidixic acid was 88.9 % which is similar to several aforementioned reports [51, 64, 68, 70]. However, other studies found either low resistance [71, 72] or none at all [65]. Resistances to tetracycline was higher (77.8 %) than previously reported (32.0 %) [72], but it was lower than in isolates recovered from conventionally grown turkeys [68].

The gene loci responsible for antibiotic resistance were detected in all resistant isolates to ciprofloxacin and erythromycin and 66.7 % of resistant isolates to tetracycline. Ciprofloxacin resistance among *C. jejuni* and *C. coli* isolates was conferred by threonine-to-isoleucine mutation of amino acid 86 of the *gyr*A protein (Thr-86-Ile), a finding that is in agreement with other previous studies [73–76]. Tetracycline resistance was attributed to the presence of the *tet*(O) gene [51]. All resistant isolates in this study were carrying *tet*(O); none of the susceptible isolates gave a positive result using specific PCR.

Conclusions

To the best of our knowledge we present here the first detailed characterization of Vietnamese *Campylobacter* isolates regarding genetic diversity, virulence-associated genes and antibiotic susceptibility. The limitation of our study is the small number of isolates. Further studies are needed to improve our knowledge about the epidemiology and relevance of *Campylobacter* for human health in Vietnam.

Authors' contributions

TNMN, HH, HE, HTT and HMH participated in the conception and design of the study and TNMN, HTT, HH and HE performed the farm and laboratory work. TNMN, HH, HE HN and HMH analyzed the data and wrote the manuscript. TNMN, HTT, HH, HE, HT, MHL, HN and HMH contributed to the analysis and helped in the manuscript discussion. All authors read and approved the final manuscript.

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Competing interests
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CHAPTER 4

Antimicrobial resistance of *Campylobacter* isolates from small scale and backyard chicken in Kenya

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Antimicrobial resistance of *Campylobacter* isolates from small scale and backyard chicken in Kenya

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Abstract

Background: Thermophilic *Campylobacter* species are a major cause of bacterial foodborne diarrhoea in humans worldwide. Poultry and their products are the predominant source for human campylobacteriosis. Resistance of *Campylobacter* to antibiotics is increasing worldwide, but little is known about the antibiotic resistance in *Campylobacter* isolated from chicken in Kenya. In this study, 35 suspected *Campylobacter* strains isolated from faeces and cloacal swabs of chicken were tested for their susceptibility to seven antibiotics using a broth microdilution assay and molecular biological investigations.

Results: Overall, DNA of thermophilic *Campylobacter* was identified in 53 samples by PCR (34 *C. jejuni*, 18 *C. coli* and one mix of both species) but only 35 *Campylobacter* isolates (31 *C. jejuni* and 4 *C. coli*) could be re-cultivated after transportation to Germany. Isolates were tested for their susceptibility to antibiotics using a broth microdilution assay. Additionally, molecular biological detection of antibiotic resistance genes was carried out. *C. jejuni* isolates showed a high rate of resistance to nalidixic acid, tetracycline and ciprofloxacin of 77.4, 71.0 and 71.0 %, respectively. Low resistance (25.8 %) was detected for gentamicin and chloramphenicol. Multidrug resistance in *C. jejuni* could be detected in 19 (61.3 %) isolates. Resistance pattern of *C. coli* isolates was comparable. Resistance to ciprofloxacin was confirmed by MAMA–PCR and PCR–RFLP in all phenotypically resistant isolates. The *tet*(O) gene was detected only in 54.5 % of tetracycline resistant *C. jejuni* isolates. The *tet*(A) gene, which is also responsible for tetracycline resistance, was found in 90.3 % of *C. jejuni* and in all *C. coli* isolates. Thirteen phenotypically erythromycin-resistant isolates could not be characterised by using PCR–RFLP and MAMA–PCR.

Conclusions: To the best of our knowledge, this study is the first report about resistance to antibiotics in thermophilic *Campylobacter* originating from chicken in Kenya. *Campylobacter* spp. show a high level of resistance to ciprofloxacin, nalidixic acid and tetracycline but also a remarkable one to chloramphenicol and gentamicin and they are multidrug resistant. Resistance to antibiotics is a global public health concern. In Kenya, resistance surveillance needs further attention in the future. Efforts to establish at least a National Laboratory with facilities for performing phenotypic and genotypic characterization of thermophilic *Campylobacter* is highly recommended.

Keywords: *Campylobacter*, Antibiotic resistance, Microdilution, Chicken, Kenya

Background

Thermophilic *Campylobacter* (*C.*) species have become the most frequent cause of bacterial gastroenteritis in humans worldwide [1]. Campylobacteriosis exceed the total number of those caused by *Salmonella*, *Shigella*, and *Escherichia coli* O157:H7 in humans, recently [2]. *Campylobacter* infections are normally self-limiting in adults but can cause diarrhoea or even mortality in children in developing as well as in developed countries [3, 4].

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A study from western Kenya showed that 20 % of patients with diarrhoea were infected by *Campylobacter* but in the group of children below 5 years *Campylobacter* was represented with 42 % [5].

Commercial poultry and free-living birds are natural reservoirs of thermophilic campylobacters. The organism has been isolated from numerous bird species, including *Columbiformes* and domestic and free-living *Galliformes* and *Anseriformes*. *C. jejuni* has been found in all areas of commercial poultry production [6, 7]. Prevalence rates in poultry, especially in slaughter-age broiler flocks, could reach 100 % on some farms. Although, *Campylobacter* is insignificant for poultry health, it is a predominant cause of foodborne gastroenteritis in humans worldwide, and contaminated poultry meat is recognized as the main source of human infections [7, 8]. In general, the knowledge about *Campylobacter* in Kenya is limited. Most of the published reports describe prevalence and antibiotic resistance in *Campylobacter* of human origin [4, 9–11]. Other reports gave information about *Campylobacter* as cause of foodborne diseases [12] and contamination of raw chicken and beef from butcheries and markets in Nairobi [13]. Information on thermophilic *Campylobacter* of animal origin from Kenya is lacking.

Resistance against antibiotics in bacteria is of public health concern. Most commonly used drugs in treatment of campylobacteriosis in humans are erythromycin, fluoroquinolones or tetracycline [14]. Although, this antimicrobial treatment is usually not necessary, however the misuse of antibiotics is widespread in Kenya [5]. Attention on resistance of *Campylobacter* is raising and warning has been launched not to misuse antibiotics such as macrolides, fluoroquinolones or alternative drugs [15]. Kenyan *Campylobacter* isolates from humans showed a high resistance rate against erythromycin (52 %), but only low resistance to ciprofloxacin, tetracycline and nalidixic acid with 6, 18 and 26 % in the past, respectively [5].

Clinical breakpoints of *Campylobacter* susceptibility based on epidemiological cut-off (ECOFF) values were recommended. EUCAST MIC distributions incorporate human and veterinary clinical data from several sources worldwide [16]. The method of choice for testing antibiotic susceptibility and determination of minimum inhibitory concentration (MIC) values of *Campylobacter* isolates is the broth microdilution assay [17, 18].

In addition to phenotypical determination of antibiotic resistance, genetic analysis of resistance determinants in *Campylobacter* can be carried out. A replacement of threonine by isoleucine at amino acid 86 in the *gyr*A gene [19, 20] and a mutation at position 2074 and 2075 on the 23S rRNA gene are the main mechanisms for fluoroquinolone and erythromycin resistance, respectively [21]. Presence of *tet*(O) and/or *tet*(A) genes is responsible for tetracycline resistance [22]. A mismatch amplification mutation assay (MAMA–PCR) can be used for detection of the mutations in *gyr*A and 23S rRNA genes in *C. coli* and *C. jejuni* responsible for ciprofloxacin and erythromycin resistance, respectively [21, 23]. PCR-restriction fragment length polymorphism (PCR–RFLP) technique [24] is available for detection of erythromycin resistance as well as specific PCR assays for *tet*(O) and *tet*(A) genes. These methods allow the investigation of antibiotic resistance of *Campylobacter* even in samples from which no *Campylobacter* could be isolated.

To the best of our knowledge there is no report available about antibiotic resistance of thermophilic *Campylobacter* species isolated from chicken in Kenya. MICs and results of molecular assays on the resistance of recent Kenyan *C. coli* and *C. jejuni* are presented.

Methods

Sample collection and *Campylobacter* isolation

In total, 35 geographically different native breed layer flocks were sampled. The chickens were housed in backyards and homesteads of small scale farmers from the outskirts of Thika, a town 40 km northeastern of Nairobi, Kenya. Farmers kept between 10 and 1000 layers. The birds were fed on commercially formulated ration from different sources and sometimes supplied with the leftover and residual food. All the manufactures used antibiotics as part of the ingredients in the feed. During the rearing of these chickens, antibiotics were used for prevention and treatment of diseases without any instructions. Ten to 30 cloacal swabs and faecal samples were collected from each flock according to flock size. *Campylobacter* were isolated in Kenya Medical Research Institute, Nairobi according to the guidelines of ISO 10272-1 [25]. The isolates were preserved in 1.5 ml Eppendorf tubes filled with skimmed milk medium for 1-week transportation from Kenya to Friedrich–Loeffler-Institut, Jena, Germany for further laboratory analysis. *Campylobacter* strains were re-cultivated on both Mueller–Hinton agar and CCDA (Oxoid GmbH, Wesel, Germany) under microaerophilic conditions (5 % O_2, 10 % CO_2, and 85 % N_2) at 37 °C for 48–72 h.

DNA extraction

DNA from viable bacteria was extracted using the High Pure PCR Template Preparation Kit™ (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Skimmed milk samples of *Campylobacter* that could not be re-cultivated were treated with phenol–chloroform to extract DNA. Briefly, 500 µl of skimmed milk medium was boiled for 5 min. After cooling the liquid was mixed with 500 µl buffer-saturated phenol (Carl Roth GmbH, Karlsruhe, Germany)

and centrifuged for 5 min. at 13,400 rpm (miniSpin, Eppendorf, Hamburg, Germany). 500 µl chloroform/isoamyl alcohol (24:1 vol/vol) was added to the aqueous phase, mixed and centrifuged for 5 min. at 13,400 rpm. DNA from the aqueous phase was precipitated by mixing with 0.6 volume of isopropanol at room temperature. After centrifugation, the supernatant was discarded and the DNA was air dried and finally dissolved in 50 µl 10 mM Tris (Carl Roth GmbH).

Multiplex PCR for identification of *Campylobacter* species

A mPCR assay was used to identify thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*) as described by El-Adawy et al. [26]. Briefly, 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 mM each; Carl Roth GmbH), 2.0 µl of each primer (Jena Bioscience GmbH), 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 programme: one cycle of 1 min at 96 °C was followed by 35 cycles each consisting of 60 s at 95 °C of denaturation, 90 s at 59 °C of annealing, and 60 s at 72 °C of elongation. The PCR was terminated after 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.

Antimicrobial susceptibility testing and determination of MICs

The antimicrobial susceptibility of *C. jejuni* and *C. coli* isolates was tested against seven antibiotic agents (chloramphenicol, erythromycin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, and tetracycline) using the Sensititre™ Campylobacter plates—EUCAMP (Trek Diagnostic Systems Ltd., East Grinstead, UK). The MIC values were detected using different concentration ranges as previously described [23]. Briefly, *Campylobacter* isolates grown on Mueller–Hinton agar (Oxoid GmbH) supplemented with 10 % bovine blood under microaerophilic conditions were suspended in NaCl solution (0.9 %) to obtain a turbidity corresponding to a McFarland standard of 0.5 (Dr. Lange, CADAS photometer 30, Berlin, Germany). One-hundred and fifty milliliters of the above suspension were diluted with 10 ml Mueller–Hinton broth (Oxoid GmbH) resulting in a concentration of approximately 10^6–10^7 colony forming units (cfu)/ml. One hundred milliliters of the inoculum was filled in each well of the plate; the plates were sealed and incubated at 37 °C for 24 h under microaerophilic conditions. Results were read either visually or photometrically (Tecan Deutschland GmbH, Crailsheim, Germany) using the computer program easyWIN fitting (version V6.1, 2000). *C. jejuni* DSM 4688 and *C. coli* DSM 4689 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were included in each batch of broth microdilution assay for quality control. The lowest concentration of antibiotics that prevents visible growth of the microorganism is defined as the MIC.

Molecular biological detection of antibiotic resistance determinants

Extracted *Campylobacter* DNA from all samples and strains was used for molecular biological determination of selected antibiotic resistance determinants by PCR.

Erythromycin resistance

Detection of mutations at positions 2074 and 2075 in domain V of the 23S rRNA gene, which mediates resistance to erythromycin, was carried out by MAMA–PCR and PCR–RFLP as described previously [21, 24]. Genes responsible for resistance of erythromycin and ciprofloxacin were tested at two loci using MAMA–PCR and PCR–RFLP. Primers and their sequences are given in Table 1.

Ciprofloxacin resistance

A single point mutation (Thr-86-Ile) in the quinolone resistance-determining region (QRDR) of *gyr*A gene was defined as source of high-level resistance to fluoroquinolones [23]. MAMA–PCR for *C. jejuni* isolates was carried out as described previously [27], for *C. coli* a procedure according to Zirnstein et al. [28] was used. Primer details are given in Table 1.

Tetracycline resistance

Primers DMT1 and DMT2 (Jena Bioscience GmbH) were used for the detection of the *tet*(O) gene which is strongly associated with tetracycline resistance in *C. jejuni* and *C. coli* as described previously [29]. As a second gene locus associated with tetracycline resistance the presence of *tet*(A) was examined by a previously described PCR assay [22]. An alternative, in-house validated PCR assay was created based on *tet*(A) sequences (GenBank acc. no.

Table 1 List of primers and primer sequences used for detection of antimicrobial resistance genes

Antibiotic	Method	Primer	Sequence (5′–3′)	Amplicon length (bp)	Reference
Erythromycin	MAMA–PCR[a]	23SRNA-F ERY2075-R ERY2074-R	TTA GCT AAT GTT GCC CGT ACC G TAG TAA AGG TCC ACG GGG TCG C AGT AAA GGT CCA CGG GGT CTG G	485	[17]
	PCR–RFLP[a]	F2-campy-23S R2-campy-23S	AAT TGA TGG GGT TAG CAT TAG C CAA CAA TGG CTC ATA TAC AAC TGG	316	[20]
Ciprofloxacin	MAMA–PCR[b]	CampyMAMAgyrA1 CampyMAMAgyrA5	TTT TTA GCA AAG ATT CTG AT CAA AGC ATC ATA AAC TGC AA	265	[19]
	MAMA–PCR[c]	GZgyrACcoli3F CanpyMAMAgyrA8	TAT GAG CGA TAT TAT CGG TC TAA GGC ATC GTA AAC AGC CA	192	[24]
Tetracycline	*tet*(O) PCR	DMT 1 DMT 2	GGC GTT TTG TTT ATG TGC G ATG GAC AAC CCG ACA GAA GC	559	[25]
	tet(A) PCR	Tet(A)-F Tet(A)-R	GTG AAA CCC AAC ATA CCC C GAA GGC AAG CAG GAT GTA G	888	[18]
	tet(A) PCR	tet-A-1 tet-A-2	GCT CAC GTT GAC GCA GGA AAG ATC GTC ATT GTC CGT TAC	486	This study

[a] 23S rRNA gene mutation

[b] *gyr*A gene mutation *Campylobacter jejuni*

[c] *gyr*A gene mutation *Campylobacter coli*

JX891463 and JX891464)). Briefly, primers tet-A-1 and tet-A-2 (Table 1; Jena Bioscience GmbH) were used with the following PCR programme: An initial denaturation at 96 °C for 60 s was followed by 35 cycles of denaturation (96 °C for 15 s), annealing (49 °C for 60 s) and extension (72 °C for 30 s). PCR was terminated by final extension at 72 °C for 60 s. The PCR resulted in a 486 bp product.

All PCR products were analyzed by electrophoresis on 1.5 % agarose gels, staining with ethidium bromide and visualization under UV light.

DNA sequencing

PCR products obtained by *tet*(A) PCRs were sequenced by cycle sequencing with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the instructions of the manufacturer. In addition to the amplification primers of the Tet(A)-F/R fragment tet-A-A (5'-AAT TTT CTT CAA ATA AGG-3') and tet-A-B (5'-GTC ATT CTT ATA TTA AGT GG-3') were used as sequencing primers. Sequencing products were analyzed with an ABI PRISM 3130 genetic analyzer.

MALDI-TOF mass spectrometry

Cultured bacteria were suspended in 300 µl of bi-distilled water and mixed with 900 ml of ethanol (Carl Roth GmbH). Further treatment of samples and measurement were described by El-Ashker et al. [30].

Results

Identification of bacteria

In total, 58 isolates suspected as *Campylobacter* were recovered from faeces and cloacal swabs of chicken flocks in Kenya. After storage in skimmed milk medium and transportation to Germany only 40 of these isolates could be re-cultivated. Four *C. coli* and 31 *C. jejuni* were identified by mPCR (Table 2). Five other isolates were identified by MALDI-TOF mass spectrometry as members of genera *Bacillus*, *Staphylococcus*, *Ochrobactrum* as well as two *Bordetella* isolates.

Eighteen skimmed milk tubes contained *Campylobacter* DNA [14 *C. coli*, 3 *C. jejuni* and one sample harboured both *C. coli* and *C. jejuni* (Table 2)].

Antimicrobial susceptibility profiles and multidrug resistance

The results of antimicrobial susceptibility testing of *C. jejuni* and *C. coli* isolates and the rate of resistance to seven antimicrobial agents are given in Tables 3 and 4, respectively. The *C. jejuni* isolates showed a high rate of resistance to nalidixic acid, tetracycline and ciprofloxacin with 77.4, 71.0 and 71.0 %, respectively. Low resistance rates were detected for gentamicin and chloramphenicol, both with 25.8 % of the isolates. For the low number of *C. coli* isolates (n = 4) a similar pattern was observed. Only two isolates were susceptible to all tested antimicrobial agents, one isolate was resistant to all tested antibiotics.

The multidrug resistance profiles of 31 *C. jejuni* isolates are shown in Table 5. Multidrug resistance to three or more classes of antibiotics was found in 19 isolates (61.3 %) and was observed in a range between 5.3 and 26.3 %.

Molecular biological detection of antibiotic resistance determinants

DNA of 35 viable *Campylobacter* isolates and of 18 non-growing samples was investigated by PCR to detect antibiotic resistance. Mismatch amplification mutation assay

Table 2 Results of cultivation and multiplex PCR identification of *Campylobacter* isolates

Cultivation	mPCR identification of *Campylobacter*				Total n (%)
	C. jejuni	*C. coli*	Not identified	*C. coli/C. jejuni*	
Positive (n)	31	4	5	0	40 (69.0)
Negative (n)	3	14	0	1	18 (31.0)

(MAMA–PCR) was used to characterize a *gyr*A gene mutation associated with ciprofloxacin resistance as well as mutations in 23S rRNA genes as cause of erythromycin resistance. The molecular biological detection of resistance to ciprofloxacin in both *C. coli* and *C. jejuni* was also confirmed by change of amino acid 86 from threonine to isoleucine in the *gyr*A gene. Additional to the ciprofloxacin resistant *Campylobacter* isolates (Tables 3, 4), three *Campylobacter* DNAs were detected harbouring the *gyr*A gene mutation. The results were confirmed by PCR–RFLP according to Vacher et al. [24]. Mutations at positions 2074 and 2075 of the 23S rRNA genes in 13 phenotypically erythromycin-resistant isolates could neither be detected by using PCR–RFLP nor MAMA–PCR.

The *tet*(O) gene which is mainly responsible for tetracycline-resistance was detected by PCR in 12 out of 22 resistant *C. jejuni* isolates (54.5 %) and in all tetracycline resistant *C. coli* isolates. *tet*(O) gene was not detected in DNA extracted from the non-growing samples. Additionally, a newly developed PCR assay was used for the detection of the *tet*(A) gene. *tet*(A) was detected in 28 out of 31 *C. jejuni* (90.3 %) and in all 4 *C. coli* isolates. In 3 out of 14 non-growing samples which harboured *C. coli* DNA, *tet*(A) gene could be found as well as in the one sample where both *C. jejuni* and *C. coli* were detected.

Table 3 Results of MIC determination and resistance rates of Kenyan *Campylobacter jejuni* isolates

Class	Antibiotic (µg/ml)	Antibiotic susceptibility of *Campylobacter jejuni* (n=31)											R* (%)
		0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
Macrolides	Chloramphenicol						9	3	6	5	***8***		25.8
	Erythromycin				14			1	***2***	***2***	***12***		51.6
Fluoroquinolones	Ciprofloxacin	5	2	2		***2***	***1***	***19***					71.0
Quinolones	Nalidixic acid							4	1	2	***2***	***22***	77.4
Aminoglycosides	Gentamicin		2	1	4	11	5	***2***	***1***	***5***			25.8
	Streptomycin					12	2	4	***1***	***12***			41.9
Tetracyclines	Tetracycline			6	1	2		***1***	***1***	***20***			71.0

Boldface in italic type indicates the number of resistant isolates. A thick black line indicates the break point between clinically sensitive and resistant strains

R* resistance rate

Table 4 Results of MIC determination and resistance rates of Kenyan *Campylobacter coli* isolates

Class	Antibiotic (µg/ml)	Antibiotic susceptibility of *Campylobacter coli* (n=4)											R* (%)
		0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
Macrolides	Chloramphenicol						2				***2***		50.0
	Erythromycin				4								0
Fluoroquinolones	Ciprofloxacin	1						***3***					75.0
Quinolones	Nalidixic acid								1			***3***	75.0
Aminoglycosides	Gentamicin		1		1	1		***1***					25.0
	Streptomycin					2		2					0
Tetracyclines	Tetracycline			1	1	1				***1***			25.0

Boldface in italic type indicates the number of resistant isolates. A thick black line indicates the break point between clinically sensitive and resistant strains

R* resistance rate

Table 5 Multidrug resistance profiles of 19 *Campylobacter jejuni* isolates

Antibiotic resistance profile	No. of resistant isolates (%)
TET, CIP, NAL, ERY, GEN, STR, CHL	1 (5.3)
TET, CIP, NAL, ERY, STR, CHL	1 (5.3)
TET, CIP, NAL, ERY, GEN, STR	3 (15.8)
TET, CIP, NAL, GEN, STR, CHL	1 (5.3)
TET, CIP, NAL, ERY, STR	2 (10.5)
TET, CIP, NAL, ERY, CHL	1 (5.3)
TET, CIP, NAL, ERY	2 (10.5)
TET, CIP, NAL, STR	1 (5.3)
TET, CIP, NAL	5 (26.3)
CIP, NAL, ERY, STR, CHL	1 (5.3)
CIP, NAL, STR	1 (5.3)

TET tetracycline, *CIP* ciprofloxacin, *NAL* nalidixic acid, *ERY* erythromycin, *GEN* gentamicin, *STR* streptomycin, *CHL* chloramphenicol

Discussion

The antimicrobial susceptibility patterns among *Campylobacter* isolates originating from chicken in Kenya were analyzed according to the guidelines of CLSI for *Enterobacteriaceae* which had been guided by previous reports [17, 31–34]. Clinical breakpoints for interpretation of MIC values of *C. jejuni* and *C. coli* from chicken are available [31, 35]. In this study a commercially available broth microdilution assay was used for the determination of MIC values for seven antibiotics. The assay already proved to be suitable in previous investigations [21, 27, 34, 36, 37].

In this study, only 40 out of 58 suspected *Campylobacter* samples could be re-cultivated. The storage conditions (temperature, microaerophilic atmosphere) using skimmed milk medium were possibly not ideal. However, it had been demonstrated that *C. jejuni* can survive up to 14 days at 1 °C or 2.5 days at 20 °C in sterile skimmed milk [38, 39]. Alternative storage of *Campylobacter* cultures using transport medium (for example Amies medium) or cryovials is recommended for future investigations.

In 53 out of 58 collected samples, *Campylobacter* DNA was identified by mPCR assay [26]. The majority of the cultures proved to be *C. jejuni* (88.6 %) which is in agreement with previous studies in chicken [6, 7]. In 18 DNA extracts of non-viable samples, 3 *C. jejuni* (16.7 %), one mixed population of *C. jejuni/C. coli* and 14 *C. coli* were identified. These findings are in agreement with those of a previous study that found a longer viability of *C. jejuni* in comparison to *C. coli* in biological milieu [40]. It may be possible that some *C. coli* isolates had been lost during the shipment period. In summary, *C. jejuni* was identified much more often than *C. coli* (64.2 %) by mPCR investigation. In agreement with other studies, the findings highlighted the usefulness of mPCR as a reliable, sensitive, time and cost saving method for identification of thermophilic *Campylobacter* [26].

The antibiotic susceptibility of 35 *Campylobacter* isolates from Kenyan chicken was investigated using European Committee on Antimicrobial Susceptibility Testing and epidemiological cut-off values (EUCAST–ECOFFS) [16]. A broth microdilution assay was used as a standardized, easy, and reliable method for the determination of MIC of seven antibiotics [17, 31–34]. High resistance rates were obtained for ciprofloxacin, nalidixic acid and tetracycline with more than 70 % which is in agreement with a recent European Food Safety Authority (EFSA) report [41]. These results are in contrast to those of Brooks et al. [5] who reported resistance rates for *Campylobacter* recovered from humans with diarrhoea in Western Kenya for ciprofloxacin, nalidixic acid and tetracycline with 6, 26 and 18 % in 2006, respectively. The general high rates of resistance in the chicken isolates may be caused by availability and uncontrolled use of antibiotics by small farmers [42].

Resistance to chloramphenicol is remarkable with 25.8 % in this investigation. Use of chloramphenicol is banned in animal breeding in Europe for more than 20 years, but still it is often used in many third world countries [43]. It is easy to obtain antibiotics over-the-counter and other unregulated venues and injudicious use promotes the development of resistance to antimicrobial agents. Resistance to gentamicin in the isolates obtained from chicken was low in this study (25.8 %), but *Campylobacter* isolated from broilers and turkeys were totally susceptible to gentamicin [37, 41, 44, 45]. Erythromycin resistance rates found in this study correspond to those of similar studies elsewhere [41, 44, 45].

Multidrug resistance was detected in 61.3 % of the *Campylobacter* isolates. Eleven different combinations were found (Table 5). Frequent, resistance to ciprofloxacin, nalidixic acid and tetracycline was identified (17 out of 19 multidrug resistant isolates) which is in agreement with previous investigation using Vietnamese *Campylobacter* isolates [45]. However, EFSA [41] reported low level of multidrug resistance in *C. jejuni* from broilers of the member states of the EU.

The emerging of antibiotic resistance has been attributed to the overuse and misuse of antimicrobial agents in both the developed and developing world. Antibiotics are widely used as growth supplements in livestock and to prevent infections [46]. The emerging of multidrug resistance may reflect acquisition of different resistance determinants on the same DNA molecule or single determinants, such as multidrug pumps, that specify efflux activity against different antimicrobial agents [47]. The mechanisms of genetic resistance might be chromosomal or plasmid-borne, and represent a combination of endogenous and acquired genes. In general, mechanisms of

antibiotic resistance as modification of the antibiotic by aminoglycoside-modifying enzymes (AphA, AadE, Sat), enzymatic inactivation of the antibiotic by β-lactamase and modification of the DNA gyrase target, mutations in 23S rRNA genes were included for aminoglycosides, beta-lactams, fluoroquinolones, macrolides and tetracyclines, respectively [48, 49]. The multidrug efflux pump CmeABC has been involved in the resistance mechanisms of *C. jejuni* and *C. coli* to tetracyclines, fluoroquinolones, macrolides and beta-lactams [49].

Molecular biological methods were used for detection of antibiotic resistance determinants either using DNA isolated from cultures or that of non-cultured bacteria [27, 37]. All isolates of this study which were resistant to ciprofloxacin carried a mutation of the amino acid 86 of the *gyr*A resulting in a change from threonine to isoleucine. This mutation was detected also in 3 DNA samples extracted from skimmed milk. The MAMA–PCR protocol allowed the detection of the *gyr*A mutation and PCR–RFLP was confirming the mutation from (ACA to ATA) of amino acid 86. This result was in agreement with previous reports showing that both methods are simple, reliable, rapid tools that can be used as screening methods [27, 37]. In *Campylobacter*, resistance to erythromycin is chromosomally encoded by an alteration of the 23S rRNA gene. High level resistance to erythromycin is caused by mutations at position 2074 and/or 2075 of the domain V of this gene. In this study the mutations were neither detected by MAMA–PCR nor by PCR–RFLP.

The *tet*(O) gene is known to be responsible for tetracycline resistance in *Campylobacter* isolates [29]. In this study, only 54.5 % of the tetracycline resistant isolates harboured the *tet*(O) gene. The *tet*(A) gene also plays role in resistance to tetracycline [22]. The efflux gene *tet*(A) is coding for an approximately 46 kDa membrane-bound efflux protein for membrane-associated proteins and is involved in the export of tetracycline from the cell [50]. In this study, using the recommended primers for *tet*(A) amplification [18] PCR products of 696 bp instead of 888 bp were obtained. DNA sequencing of amplicons and database search resulted in 99.0 % homology to a partial putative integral membrane protein and a putative periplasmic protein. Hence, a new PCR assay based on *tet*(A) gene sequences for *C. jejuni* (acc. no. JX891464) and *C. coli* (acc. no. JX891463) was developed. Parameters such as limit of detection, limit of quantification, PCR efficiency and specificity were considered during an in-house validation process. Amplicon length was 486 and the amplicons were sequenced to confirm the identity. The *tet*(A) gene was much more frequently identified in the Kenyan *Campylobacter* isolates than *tet*(O) (35 vs 13).

To the best of our knowledge this is the first report on the status of antibiotic susceptibility of thermophilic *Campylobacter* from chicken in Kenya. High level of resistance to ciprofloxacin, erythromycin and nalidixic acid as well as multidrug resistance was detected previously in Kenya. In Kenya, this problem is reported to be caused by the increasing rate of unregulated over-the-counter sale without prescriptions of these antibiotics, mainly to humans self-treatment of suspected infections and to a lesser extent for use in animals [51]. These findings also demonstrate the potential for antibiotic-resistant bacteria to spread through the food chain from animals treated with antibiotics for humans. Such misuse and overuse may have resulted in the selection of resistant mutants or acquisition of antibiotic resistance genes from other organisms through the process of genetic exchange.

It is recommendable that a long-term local surveillance programme is adopted for monitoring changes in resistance among *Campylobacter* isolates. Efforts to establish at least a National Laboratory with facilities for performing phenotyping and genotyping methods is highly recommended. Emphasis should be given on educational advertising to reduce the input of antibiotics in animal breeding to minimize the potential hazard for humans.

Authors' contributions

TNMN, HH, JN, HE, HT and HMH participated in the conception and design of the study. TNMN, HH, JN, JM and HE performed farm and laboratory work. TNMN, HH, JN, HE, HT, HN and HMH analysed the data and wrote the manuscript. All authors contributed to the analysis and supported the manuscript discussion. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The data supporting the findings of this study are contained within the manuscript.

Ethics approval and consent to participate

This study was carried out in strict accordance with the recommendations of the National Guidelines on the Care and Use of Animals in Research, Education and Training in Kenya, Consortium for National Health Research (CNHR) which complies with the international laws and regulation regarding ethical considerations, transport, housing and experimental use of animals in research.

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CHAPTER 5

GENERAL DISCUSSION

Discussion

Thermophilic *Campylobacter* are the most common bacterial cause of gastroenteritis in humans worldwide. Poultry and poultry products are incriminated as the main sources for human infections. Epidemiological data concerning campylobacteriosis in many developing countries are limited. Consequently, it is difficult to accurately assess the burden of *Campylobacter* infections for these countries.

The review article on *Campylobacter* in South-East Asia including Cambodia, Laos and Vietnam showed that thermophilic *Campylobacter* are neglected foodborne pathogens in this area. Campylobacteriosis is a problem of public health concern in these countries and currently limited epidemiological data are existed. This is influenced by the limited number of laboratory facilities and lack of equipment and awareness of physicians and veterinarians.

In the 2nd chapter a review gives an overview about articles and reports on *Campylobacter* and campylobacteriosis in Cambodia, Laos and Vietnam. One topic in the literature is the prevalence of thermophilic *Campylobacter* in humans and their role in diarrhoea. In Vietnam a prevalence up to 11% in patients was reported. It was even higher in Cambodia and Laos. Especially, children under five years of age were affected. Animals and food as source for human infections play an important role. Carriage of *Campylobacter* by different animal species and contamination rate of meat are generally high and can reach more than 70%. Resistance to antibiotics is of public health concern, too. High rates of resistance to nalidixic acid, erythromycin, tetracycline and ciprofloxacin were detected in up to 100% of investigated isolates.

Different epidemiological characteristics of *Campylobacter* isolates for the different geographic areas (Vietnam vs Kenya) could be demonstrated (Chapter 3 and Chapter 4).

Chicken and pork meat samples from a market in Hanoi were investigated for the presence of thermophilic *Campylobacter*. *C. jejuni* and *C. coli* were detected as contaminants of chicken and pork meat. 13.3% of the samples were *Campylobacter* positive in this study (Chapter 3), which is much lower compared to 52.3% of chicken carcasses in slaughterhouses and nearly 40% of raw meat in retail contaminated with these organisms in Germany as a reported by BVL (2015). Reasons are might be that chicken and pigs are slaughtered in low numbers in Vietnam and often directly on-site, and consequently the risk of contamination during the

slaughtering process is low and generation of aerosols in slaughterhouses maybe less common.

To understand the biological characteristics of Vietnamese *Campylobacter* isolates, genotyping was applied including *fla*A-RFLP typing, MLST and microarray analysis. Eight isolates were identified as *C. jejuni* and one as *C. coli* by mPCR. *fla*A-RFLP as a rapid and simple typing method illustrated a remarkable heterogeneity. Four different strain types were detected by *Dde*I digestion of the amplified *fla*A gene. This enzyme was used because it showed the highest discriminatory power in former investigations (El-Adawy et al., 2013) in comparison with *Alu*I or *Sau*3AI. The digestion pattern of *C. coli* isolate 09CS0051 was completely different. Limitations of this typing method are the use of only a very small part of the *Campylobacter* genome and difficulties to standardize the analytical process. This complicates an inter-laboratory comparison of results.

The microarray assay was used as hybridization-based comparative genomic fingerprinting assay and confirmed the heterogeneity of the *C. jejuni* isolates. SplitsTree analysis of the hybridization results showed high genetic diversity as no isolate was identical with another one. One *C. coli* isolate was clearly distinct from the *C. jejuni* isolates. Microarray analysis confirmed heterogeneity of prevalent genotypes circulating in Vietnam.

Additionally, sequence types and clonal complexes of the isolates were determined by MLST. Six sequence types of *C. jejuni* isolates were detected. These sequence types were compared with the database on the *Campylobacter* MLST homepage (http://pubmlst.org/*Campylobacter*). ST 2837 and ST 4395 belonged to clonal complex ST-353 whereby 09CS0047 (ST 4395) was identical with an isolate which was recovered from a stool sample of a hospital inpatient with gastroenteritis in Vietnam in 2010. 09CS0049 and 09CS0066 belonged to CC ST-443. Sequence type 5799 was previously isolated from human stool samples in Japan. Three isolates could not be assigned to any known clonal complexes. The sequence type of isolate 09CS0068 had previously been discovered once in a human stool sample in Thailand, two others have not described yet. Isolate 09CS0067 represented sequence type 4099. This type belongs to the ST-460 complex and was previously identified in a human sample in Canada. *C. coli* isolate 09CS0051 belonged to sequence type 860 and ST-828. Identical isolates were found several times during the last two decades in Europe and the USA. These results are typical for strains of the Asian region but comprehensive data for Asia. A route of infection of *Campylobacter* from meat to humans can be assumed.

Furthermore, isolates were characterized regarding virulence factors associated with adhesion and invasion of host cells by PCR. All isolates harboured flagellin genes *fla*A, *fla*B, *flh*A and *fli*M. Molecular genetic approaches with defined mutants showed that *fla*A is essential for colonization. The complex flagellum of *Campylobacter* species is encoded by two tandem-oriented flagellin genes (*fla*A and *fla*B). While the function of the *fla*A gene seems to be fully elucidated, there are many speculations about the function of the *fla*B gene, which may play a role in antigenic variation or influence the motility in various environmental conditions (Wassenaar et al., 1994). *fli*Y, a gene of flagellar motor switch proteins, could not be detected. The *cia*B gene, coding for a *Campylobacter* invasion antigen, was present in most of the *C. jejuni* isolates. It was absent in 09CS0049 and *C. coli* 09CS0051. Another gene which is important in the invasion process of *Campylobacter* to host cells is *iam*A. It was detected in all *C. jejuni* isolates. Carvalho et al. (2001) described the detection of the *iam*A gene in 85% of invasive *C. jejuni* but in non-invasive isolates it is rare. Also the *cad*F gene was detected in all *C. jejuni* isolates. It encodes for an outer-membrane protein which mediates the binding of the bacteria to fibronectin (Konkel et al., 1997). Based on the results it can be concluded that these Vietnamese isolates represented invasive *C. jejuni* strains. Cytolethal distending toxin causes direct DNA damage leading to induction of DNA damage checkpoint pathways (Lee et al., 2003). In all Vietnamese *C. jejuni* strains isolated from chicken and pork meat the complete *cdt* gene cluster was observed. Only a minority of *C. jejuni* isolates gave positive PCR results for *virB*11 encoding a putative component of a type IV secretion system. The 25% prevalence of the *virB*11 gene in *C. jejuni* isolates in this study is higher than 10.3% in human isolates reported by Bacon et al. (2002) but much lower than in pig isolates (35.7%). Nevertheless, the role of the protein encoded by the *virB*11 gene in the invasion and colonization process of eukaryotic cells by *Campylobacter* species could not be elucidated.

The antimicrobial susceptibility profiles among the Vietnamese isolates were analyzed based on the guidelines of CLSI (CLSI, 2008). All isolates were sensitive to gentamicin and most of isolates (88.8%) were sensitive to chloramphenicol, erythromycin and streptomycin. In contrast to our findings, a previous study reported high resistance to streptomycin with 60.0% (Gu et al., 2009). The resistance rate to ciprofloxacin was 66.7% which is in agreement with a previous study showing high resistance (Mazi et al., 2008; Gu et al., 2009), but in contrast to another study with only 9.5% (Hariharan et al., 2009). Resistance to nalidixic acid was 88.9% which is similar to several aforementioned reports (Luangtongkum et al., 2006; Luangtongkum et al., 2007; Mazi et al., 2008; Gu et al., 2009). However, other studies found

either low resistance (Andersen et al., 2006a; Andersen et al., 2006b) or none at all (Miflin et al., 2007). Resistance to tetracycline was higher (77.8%) than previously reported (32.0%) (Andersen et al., 2006a), but it was lower than in isolates recovered from conventionally grown turkeys (Luangtongkum et al., 2006).

The gene loci responsible for antibiotic resistance were detected in all resistant isolates to ciprofloxacin and erythromycin and 66.7% of resistant isolates to tetracycline. Ciprofloxacin resistance among *C. jejuni* and *C. coli* isolates was conferred by threonine-to-isoleucine mutation of amino acid 86 of the *gyr*A protein (Thr-86-Ile), a finding that is in agreement with other previous studies (Wang et al., 1993; Charvalos et al., 1996; Ruiz et al., 1998; Wardak et al., 2005). Tetracycline resistance was attributed to the presence of the *tet*(O) gene (Mazi et al., 2008). All resistant isolates in this study were carrying *tet*(O), none of the susceptible isolates gave a positive result using specific PCR.

Our study on testing the antibiotic resistance of 35 *Campylobacter* isolates collected from small scale and backyard chicken in Kenya using European Committee on Antimicrobial Susceptibility Testing and Epidemiological Cut-off Values (EUCAST-ECOFFS, 2013) showed a high resistance rate to ciprofloxacin, nalidixic acid and tetracycline with more than 70% which is in agreement with a recent EFSA report (EFSA, 2015). These results are in contrast to those of Brooks et al. (2006) who reported resistance rates for *Campylobacter* recovered from humans with diarrhoea in Western Kenya for ciprofloxacin, nalidixic acid and tetracycline with 6%, 26% and 18% in 2006, respectively. The general high rates of resistance in the chicken isolates may be caused by availability and uncontrolled use of antibiotics by small farmers (EFSA, 2015). Resistance to chloramphenicol was remarkable with 25.8% in this investigation. Use of chloramphenicol is banned in animal breeding in Europe for more than 20 years, but it still used in many third world countries. Resistance to gentamicin was low (25.8%) in tested Kenyan isolates in this study but *Campylobacter* isolates from broilers and turkeys which were totally susceptible to gentamicin (Blaser et al., 1980; Luangtongkum et al., 2006; CLSI, 2008; EFSA, 2014; EFSA, 2015). Erythromycin resistance rates found in this study correspond to those of similar studies elsewhere (Blaser et al., 1980; Luangtongkum et al., 2006; EFSA., 2014).

Multidrug resistance was detected in 61.3% of the *Campylobacter* isolates. Frequently, resistance to ciprofloxacin, nalidixic acid and tetracycline was identified (17 out of 19 multidrug resistant isolates) which is in agreement with previous investigation using Vietnamese *Campylobacter* isolates (Luangtongkum et al., 2006). However, EFSA (2014)

reported low level of multidrug resistance in *C. jejuni* from broilers of the member states of the EU.

All isolates of this study resistant to ciprofloxacin carried a mutation of the amino acid 86 of the *gyr*A resulting in a change from threonine to isoleucine. This mutation was detected also in 3 DNAs extracted from skimmed milk samples. High level resistance to erythromycin is caused by mutations at position 2074 and/or 2075 of the domain V of this gene. The *tet*(O) gene responsible for tetracycline resistance in *Campylobacter* isolates was observed in 54.5% of isolates.

Additionally, the gene *tet*(A) in charge of tetracycline resistance was tried to amplify. Using the primer recommended for *tet*(A) amplification (Abd-Hachesoo et al., 2014) PCR products of 696 bp instead of 888 bp was obtained. DNA sequencing of amplicons and database search resulted in 99% homology to a partial putative integral membrane protein and a putative periplasmic protein but not *tet*(A) gene. Hence, a new PCR assay based on *tet*(A) gene sequences for *C. jejuni* (acc. no. JX891464) and *C. coli* (acc. No. JX891463) was developed. Amplicon length was 486 and the amplicons were sequenced to confirm the identity. The *tet*(A) gene was much more frequently identified in the Kenyan *Campylobacter* isolates than *tet*(O) gene (35 versus 13).

To the best of our knowledge these studies are the first studies on genotyping and antibiotic resistance of *Campylobacter* isolates collected from Vietnam and Kenya. The *Campylobacter* isolates from Vietnam proved their high diversity as well as antibiotic susceptibility. High antibiotic resistance of *Campylobacter* from Kenya underscore the potential for antibiotic-resistant bacteria to spread through the food chain from animals treated with antibiotics for humans.

These evidence-based findings are necessary for consideration when designing long-term local surveillance program for antibiotic resistance among *Campylobacter* isolates to reduce the hazard for humans.

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SUMMARY OF THE THESIS

Summary

Thermophilic *Campylobacter* are well recognized as the leading cause of bacterial foodborne gastroenteritis worldwide. *Campylobacter* are microaerophilic growing, Gram-negative, curved corkscrew shaped and show motility. They colonize the intestine of many wild and domestic animals, particularly that of poultry. The review on *Campylobacter* shows specific aspects of *Campylobacter*/campylobacteriosis including: (i) taxonomy of the genus *Campylobacter*, growth and survival characteristics; (ii) detection, isolation and confirmation of *Campylobacter*; (iii) poultry as natural hosts for *Campylobacter* species as zoonotic pathogens and (iv) antimicrobial resistance in *Campylobacter* (Chapter 1).

Knowledge on *Campylobacter* is limited in many developing countries, particularly in Southeast Asia. The review covering published articles from 1971 to 2016 in Cambodia, Laos and Vietnam showed them as neglected bacterial pathogens. Literature on prevalence of thermophilic *Campylobacter* in humans showed a prevalence up to 11% in Vietnam while it was even higher in Cambodia and Laos. Especially, children under five years of age were affected. Animals and food as source for human infections play an important role. Carriage of *Campylobacter* by different animal species and contamination rate of meat are generally high and can reach more than 70%. Resistance to antibiotics is of public health concern. High rates of resistance to nalidixic acid, erythromycin, tetracycline and ciprofloxacin were detected in up to 100% of isolates (Chapter 2).

Nine isolates (eight *C. jejuni* and one *C. coli*) isolated from meat were identified by multiplex PCR, and tested for the presence or absence of 29 gene loci associated with virulence, lipooligosaccharide (LOS) biosynthesis and further functions. *fla*A typing, multilocus sequence typing and investigation by microarray assay showed a high degree of genetic diversity among these isolates. In all isolates motility genes (*fla*A, *fla*B, *flh*A, *fli*M), colonization-associated genes (*cad*F, *doc*B), toxin genes (*cdt*A, *cdt*B, *sec*D, *sec*F), and LOS biosynthesis gene *pgl*B were detected. Eight gene loci could not be detected by PCR. Different gene loci for *cia*B (22.2 %), *Cje*1280 (77.8 %), *doc*C (66.7 %), and *cgt*B (55.6 %) were found. *iam*A, *cdt*C, and the type 6 secretion system were present in all *C. jejuni* isolates but not in *C. coli*. *fla*A typing resulted in five different genotypes within *C. jejuni*, MLST classified the isolates into seven sequence types. The microarray assay analysis showed also high genetic diversity within Vietnamese *Campylobacter* isolates which resulted in eight different types for *C. jejuni*. Antibiotic susceptibility profiles showed that all isolates were

sensitive to gentamicin and most isolates (88.8 %) were sensitive to chloramphenicol, erythromycin and streptomycin. Resistance rates to nalidixic acid, tetracycline and ciprofloxacin were 88.9 %, 77.8 % and 66.7 %, respectively. This is the first report that shows high genetic diversity and remarkable antibiotic resistance of *Campylobacter* strains isolated from meat in Vietnam. These strains can be considered of high public health significance (Chapter 3).

Antibiotic susceptibility of thermophilic *Campylobacter* isolates which were collected from Kenya were tested using the broth microdilution assay. Molecular biological detection of genes associated with resistance completed the results. Thermophilic *Campylobacter* was identified in 53 samples by PCR (34 *C. jejuni*, 18 *C. coli* and one mix of both species) but only 35 *Campylobacter* isolates (31 *C. jejuni* and 4 *C. coli*) could be recultivated after transportation to Germany. *C. jejuni* isolates showed a high rate of resistance to nalidixic acid, tetracycline and ciprofloxacin of 77.4 %, 71.0 % and 71.0 %, respectively. Low resistance (25.8 %) was detected for gentamicin and chloramphenicol. Multidrug resistance in *C. jejuni* could be detected in 19 (61.3 %) isolates. Resistance patterns of *C. coli* isolates were comparable. Resistance to ciprofloxacin was confirmed by MAMA-PCR and PCR-RFLP in all phenotypically resistant isolates. The *tet*(O) gene was detected only in 54.5 % of tetracycline resistant *C. jejuni* isolates. The *tet*(A) gene, which is also responsible for tetracycline resistance, was found in 90.3% of *C. jejuni* and in all *C. coli* isolates. Thirteen phenotypically erythromycin resistant isolates could not be characterized by using PCR-RFLP and MAMA-PCR. This study showed a high level of resistance to ciprofloxacin, nalidixic acid and tetracycline but also a remarkable one to chloramphenicol and gentamicin and multidrug resistance seems to be a prevalent problem. Resistance to antibiotics is of global public health concern. In Kenya, resistance surveillance needs further attention (Chapter 4).

Zusammenfassung

Genotypisierung und Antibiotikaresistenz thermophiler *Campylobacter*-Isolate aus Vietnam und Kenia

Thermophile *Campylobacter* werden als häufigste Ursache bakteriell bedingter, durch Lebensmittel verursachte Gastroenteritiden weltweit angesehen. *Campylobacter* wachsen als gekrümmte Stäbchen unter mikroaerophilen Bedingungen, sind Gram-negativ und beweglich. Sie kolonisieren den Verdauungstrakt vieler Wildvögel und den von geflügels.

Ein Überblick über *Campylobacter* zeigt verschiedene Aspekte im Komplex *Campylobacter*/Campylobakteriose, wobei folgendes berücksichtigt wird: (i) die Taxonomie der Gattung *Campylobacter* mit Wachstumsbedingungen und Überlebenscharakteristika, (ii) die Isolierung, den Nachweis bzw. die Bestätigung von *Campylobacter*-Spezies, (iii) Geflügel als natürlichen Wirt für die zoonotischen Pathogene *Campylobacter* und (iv) die Empfindlichkeit von *Campylobacter* - Isolaten gegenüber verschiedenen Antibiotika (Kapitel 1).

Der Kenntnisstand bezüglich *Campylobacter* und deren Verbreitung, Eigenschaften, Antibiotikaresistenz usw. in Entwicklungsländern ist häufig mangelhaft. Dies trifft auch auf Südostasien zu. Der Übersichtartikel über *Campylobacter* aus publizierten Beiträgen von 1971 bis 2016 zeigt diese Erreger als vernachlässigte bakterielle Pathogene in Kambodscha, Laos und Vietnam. Die Literatur bezüglich der Prävalenz thermophiler *Campylobacter* beim Menschen und als Ursache von Durchfallerkrankungen zeigt eine Prävalenzrate von bis zu 11% in Vietnam. Diese ist in Kambodscha und Laos noch höher. Besonders betroffen von Campylobakteriosen sind Kinder im Alter bis zu 5 Jahren. Umgang mit Tieren und Lebensmittel spielen bei Infektionen des Menschen eine wesentliche Rolle. Verschiedene Tierarten können die Erreger übertragen und die Kontaminationsraten von Fleisch sind generell hoch und können bis 70% erreichen. Auch die Antibiotika-Resistenz von *Campylobacter*-Isolaten hat eine große Bedeutung für den Gesundheitssektor. Hohe Resistenzraten gegenüber Nalidixinsäure, Ciprofloxacin, Erythromycin und Tetrazyklin wurden beobachtet und wurden manchmal bei 100 % der Isolate festgestellt (Kapitel 2).

Neun vietnamesische *Campylobacter*-Isolate (8 *C. jejuni* und ein *C. coli*), welche als Kontaminanten bei Fleisch von Hühnern und Schweinen gewonnen wurden, konnten durch Multiplex-PCR identifiziert werden. Sie wurden auf die An- oder Abwesenheit von 29 Genorten getestet. Diese Orte sind mit der Virulenz, der Lipooligosaccharid-Synthese und weiterer Funktionen assoziiert. Durch *fla*A-Typisierung, „Multi locus sequence typing“

(MLST) und Mikroarray-Analyse konnte ein hohes Maß an genetischer Heterogenität unter den Isolaten ermittelt werden. Alle Isolate trugen bestimmte Motilitätsgene, Kolonisierungs-assoziierte und Toxin-Gene sowie das Lipooligosaccharid- Biosynthese-Gen *pgl*B. Auch ein Typ-VI-Sekretionssystem war bei allen *C. jejuni*-Isolaten nachweisbar, nicht jedoch bei dem *C. coli*-Isolat. Acht Genorte wurden durch PCR-Untersuchungen in keinem Isolat gefunden. Mehrere Gene waren in unterschiedlichem Maße präsent. *fla*A-Typisierung ergab 5 verschiedene Genotypen für die *C. jejuni*-Isolate, MLST sieben Sequenztypen. Die Mikroarray-Analyse bestätigte die starke Heterogenität. Alle Isolate waren empfindlich gegenüber Gentamicin und auch weitgehend sensibel gegenüber Chloramphenicol, Erythromycin und Streptomycin. Die Resistenzraten gegenüber Nalidixinsäure, Tetrazyklin und Ciprofloxacin betrugen 88,9%, 77,8% und 66,7%. Dieser erste Bericht zeigte eine hohe genetische Heterogenität und eine bemerkenswerte Antibiotika-Resistenz der *Campylobacter*-Isolate, welche auf einem Markt in Hanoi, Vietnam gewonnen wurden. Die Befunde sind von öffentlichem Interesse (Kapitel 3).

Die Antibiotika-Empfindlichkeit thermophiler *Campylobacter*-Isolate, welche in bäuerlichen Geflügelhaltungen in Kenia gewonnen wurden, wurde mittels Mikrodilutionsverfahren ermittelt. Die molekularbiologische Detektion von Resistenzdeterminanten komplettierten die Resultate der phänotypischen Tests. Thermophile *Campylobacter* wurden mittels PCR in 53 Fällen identifiziert (34 *C. jejuni*, 18 *C. coli*, eine Mischkultur). Leider konnten nur 35 Isolate nach dem Transport nach Deutschland rekultiviert werden. Sie zeigten eine hohe Resistenzrate gegenüber Nalidixinsäure, Tetrazyklin und Ciprofloxacin mit 77,4%, 71, 0% und 71,0%. Eine niedrigere Resistenzrate wurde für Gentamicin und Chloramphenicol festgestellt. Resistenz gegenüber verschiedenen Antibiotikaklassen (multidrug resistance) wurde in 61,3% der Isolate gefunden. Die Resistenz gegenüber Ciprofloxacin wurde durch MAMA-PCR und PCR-RFLP bestätigt. Das *tet*(O)-Gen wurde nur in 54,5% der Tetrazyklin-resistenten Isolate gefunden. Das *tet*(A)-Gen, welches ebenfalls Resistenz gegenüber Tetrazyklin vermittelt, wurde in 90,3% der Isolate detektiert. Die Bestätigung der Erythromycin-Resistenz der phänotypisch resistenten Isolate durch MAMA-PCR und PCR-RFLP mißlang. Insgesamt wurde ein hohes Maß an Antibiotika-Resistenz bei den *Campylobacter*-Isolaten aus Kenia festgestellt, wobei vor allem die Multiresistenz der Bakterien ein Problem darstellt, welches von globalem Interesse ist. Für Kenia gilt es, Überwachungsprogramme hinsichtlich der Ausbildung von Antibiotika-Resistenzen zu etablieren (Kapitel 4).

List of publications

A) Publications in peer-reviewed journals

1. Nguyen TNM, Hotzel H, El-Adawy H, Tran HT, Le MTH, Tomaso H, Neubauer H, Hafez HM (2016) Genotyping and antibiotic resistance of thermophilic *Campylobacter* isolated from chicken and pig meat in Vietnam. Gut Pathogens 8:19.

2. Nguyen TNM, Hotzel H, Njeru J, Mwituria J, El-Adawy H, Tomaso H, Neubauer H, Hafez HM (2016) Antimicrobial resistance of *Campylobacter* isolates from small scale and backyard chicken in Kenya. Gut Pathogens 8:39.

3. Nguyen TNM, Hotzel H, El-Adawy H, Tran HT, Le MTH, Tomaso H, Neubauer H, Hafez HM (2016) Thermophilic *Campylobacter* - neglected foodborne pathogens in Cambodia, Laos and Vietnam. EcoHealth (Under Review).

B) Oral presentations

1. Nguyen TNM, Hotzel H, El-Adawy H, Tomaso H, Neubauer H, Hafez HM (2014) Genotypic and phenotypic characterization of thermophilic Vietnamese *Campylobacter* isolates. FLI Symposium of Junior Scientists, Mariensee-Germany, August-2014 (English language).

2. Nguyen TNM, Hotzel H, Njeru J, Mwituria J, El-Adawy H, Tomaso H, Neubauer H, Hafez HM (2015) Testing of antimicrobial sensitivities of *Campylobacter jejuni* and *Campylobacter coli* isolates collected from Kenya. FLI Symposium of Junior Scientists, Riems-Germany, September-2015 (English language).

C) Poster

1. Nguyen TNM, Hotzel H, Njeru J, Mwituria J, El-Adawy H, Tomaso H, Neubauer H, Hafez HM (2016) Antibiotic susceptibility and multidrug resistance of *Campylobacter* isolates collected from small scale and backyard chickens in Kenya. FLI Symposium of Junior Scientists, Jena-Germany, September-2016, DOI: 10.13140/RG.2.2.27607.06567.

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Whoever cares to learn always to find a teacher (German proverb)
When eating a fruit think of the person who planted the tree (Vietnamese proverb)

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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.

17.01.2017 Tuan Ngoc Minh Nguyen