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Review article

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Abstract – Species within the genus, *Campylobacter*, have emerged over the last three decades as significant clinical pathogens, particularly of human public health concern, where the majority of acute bacterial enteritis in the Western world is due to these organisms. Of particular concern are the species, *C. jejuni* and *C. coli*, which are responsible for most of these gastrointestinal-related infections. Although these organisms have already emerged as causative agents of zoonoses, several aspects of their epidemiology and pathophysiology are only beginning to emerge. Trends in increasing antibiotic resistance are beginning to emerge with oral antibiotics, which may be the drug of choice for when it is necessary to intervene chemotherapeutically. This review wishes to examine (i) emerging clinical aspects of the disease, such as Guillain Barré syndrome (GBS), (ii) the association between these organisms and poultry as a natural host, (iii) environmental aspects of *Campylobacter* epidemiology, (iv) the emergence of atypical campylobacters (v) emerging trends in antibiotic resistance, (vi) adoption of modern methods for the detection of campylobacters.

epidemiology / poultry / PCR / zoonosis / antibiotic resistance

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1. INTRODUCTION

Campylobacter jejuni is a major cause of foodborne illness causing human acute bacterial enteritis worldwide [8, 164]. Overall the high incidence of clinical disease associated with this organism, its low infective dose in humans [137], and its potentially serious sequelae, confirms its importance as a significant public health hazard [8, 164].

Numbers of infections have declined slightly in some parts of the world during recent years, but the overall disease burden is still significant, thus there remains an urgent need to better understand how this disease is transmitted into and within the human food chain. Such challenges are increased by the observation that an increasing number of *Campylobacter* isolates from humans and the human food chain exhibit antibiotic resistance and that antimicrobial-resistant *Campylobacter* strains cause more prolonged or more severe illness than do antimicrobial-susceptible strains.

2. HISTORICAL EMERGENCE OF *CAMPYLOBACTER*

Campylobacter spp. have long been associated with the cause of veterinary diseases, such as diarrhoea in cattle, and septic abortions in cattle and sheep. Their association with human blood cultures in the late 1950's was rare and hence *Campylobacter* spp. was deemed to be an opportunistic human pathogen. It is only in the last 30 years that these organisms have been recognised as a major cause of human illness. Campylobacters may have been observed in the stools of diarrhoeic infants in Germany as early as 1880. The first recognised identification was made by McFadyen and Stockman

in 1913 (cited in [102]) in association with abortions in sheep. Confirmatory tests were carried out by Smith in 1918 (cited in [102]) when similar organisms were isolated from aborted bovine foetuses. The organisms were originally assigned to the *Vibrio* genus, due to their spiral appearance and hence Smith named the organism *Vibrio fetus.* However, it was not until 1947 that the human infection was first associated with the microaerophilic vibrios, which was associated with a pregnancy-related infection, where the fœtus died. In 1957, the work of Elizabeth King (cited in [102]) proposed two different types of vibrios associated with enteric diseases, the first being *V. fetus* and the second was found to be thermophilic in nature. It was not until 1963 that the genus *Campylobacter* (meaning "a curved rod") was proposed as it realized that the organism could not utilize sugars and had a different G+C content to that of *Vibrio* spp. The work of King was later corroborated with the work of Dekeyser and Butzler in 1972 (cited in [102]), when isolation procedures for thermophilic campylobacters were developed. This method involved the filtering of stools samples through 0.64 μ membrane filters and inoculating the filters onto agar. This method proved too cumbersome and in 1977, Martin Skirrow from Worcester Public Health Laboratory, described a simple direct technique, involving the direct culturing of faeces onto blood agar containing vancomycin, polymyxin and trimethoprim [153–156]. Plates were incubated at 43 °C in an microaerophilic atmosphere containing 5% (v/v) O_2, 10% (v/v) CO_2 and 85% (v/v) N_2. Since then, several methodological modifications have been made, thereby allowing the universal adoption of such methods and variants of standard methods, which allow routine diagnostic clinical microbiology laboratories to attempt the isolation of campylobacters from faecal specimens.

The improved isolation methods led to the publication of the first report of the frequency of campylobacters in association with humans, thereby leading to an avalanche of epidemiological research and consequently to the realization that campylobacters have now emerged as a significant public health problem for both developed and underdeveloped countries [44, 147, 153–156, 165].

3. CLINICAL ASPECTS OF *CAMPYLOBACTER* INFECTIONS

3.1. Enteric infection

Thermotolerant campylobacters (*Campylobacter jejuni/Campylobacter coli*) constitute the most frequent cause of intestinal infections worldwide. The main symptom observed is diarrhea which can vary from limited to voluminous stools which may be watery or bloody. Another frequent digestive tract symptom is abdominal pain, whereas vomiting is uncommon. Fever, headache, asthenia, and anorexia are also present and may precede diarrhea [97]. Campylobacters are enteroinvasive bacteria which lead to colitis and, in some instances, resemble inflammatory bowel disease. When pain is the major feature of the infection, differentiation from appendicitis may be difficult. Normally the disease develops two to three days after ingestion of contaminated food and the symptoms resolve themselves within a week. In comparison to *Salmonella* or *Shigella* infections, *Campylobacter* infections are usually less acute (less fever and general symptoms) with a higher tendency toward recurrence if no treatment is given; however, they are not distinguishable without performing a coproculture. Stools remain positive for several weeks. Treatment appears to be beneficial if it is administered early enough in the course of the disease [146]. The recommended drugs are erythromycin, or amoxicillin or a fluoroquinolone or tetracycline, provided the bacterium has not acquired a resistance.

Campylobacter enteritis may occur in all age groups but clinical presentation can vary according to age. In infants, the risk of

dehydration or convulsion exists. Breast feeding protects against the clinical expression of the infection [105]. Symptoms appear during the weaning period.

In hyperexposed subjects, immunity develops and the infection becomes subclinical. It occurs in developing countries in children who are repeatedly infected but also in certain populations in Western countries, e.g. raw milk drinkers and workers in poultry abattoirs.

In contrast, a decreased immune response, as may occur in elderly people or in people whose immunity is impaired by an underlying disease (diabetes, cirrhosis, cancer, immunosuppression, HIV infection), increases the risk of developing a severe infection. In a study, the risk of *Campylobacter* infection was multiplied by 40 when subjects were HIV positive compared to controls [160].

For unknown reasons, the male gender is also an important risk factor for *Campylobacter* infection. Furthermore, a decreased gastric acidity, for example following proton pump inhibitor consumption, has been shown to be a risk factor [106]. A few local complications have been documented such as appendicitis, peritonitis, cholecystitis, hepatitis or pancreatitis but are extremely rare.

The main *Campylobacter* species involved is *C. jejuni* which is responsible for 80 to 85% of all enteric *Campylobacter* infections. *C. coli* ranks second (10 to 15%). Although the latter's source may be different, pigs being the main reservoir, this does not seem to lead to a different type of disease. The other campylobacters such as *Campylobacter lari*, *Campylobacter upsaliensis*, and *Campylobacter fetus* are more seldomly found, but vary depending on different regions of the world. *C. upsaliensis*, for example, is frequently isolated in South Africa [69].

3.2. Systemic infection

Campylobacters are invasive bacteria which may translocate and reach the blood flow. Nevertheless, the frequency of septicemia detected in the case of *Campylobacter* enteric infections remains very low (0.1%), especially when compared to those associated with *Salmonella* [156].

There is one *Campylobacter* species, *C. fetus*, which is rarely found as a cause of enteritis but is quite often isolated in systemic infections. The number of systemic infections observed with *C. fetus* indeed exceeds the number due to thermotolerant campylobacters. However, more than half of the patients harbour an underlying disease, as indicated previously. This bacteriemia induces fever and leads to metastatic localization. A number of tissues can be involved, especially the vascular endothelium (aneurism, thrombophlebitis, endocarditis), bones, joints, meninges, etc. Despite its name, *C. fetus* does not appear to induce frequent abortions in humans, only a few cases have been reported. These infections must be treated vigorously because of a bad prognosis. In a survey of more than 100 cases, death occurred in 15% of the cases, one-third being attributable to the infection, and a relapse occurred in 10%. The proposed treatment includes gentamicin and a second antibiotic, e.g. amoxicillin or Augmentin® or ciprofloxacin or imipenem, according to the location and the susceptibility profile.

3.3. Post-infectious manifestations

As with other enteropathogenic bacteria, *C. jejuni* can cause post-infectious manifestations, e.g. reactive arthritis, urticaria, erythema nodosum. Interestingly, a case of immunoproliferative small intestinal disease associated with *C. jejuni* has also been described recently [74]. These complications seldomly occur (< 1%). The most important post-infectious manifestation to be considered is Guillain-Barré syndrome [157]. This syndrome is an acute demyelinating disease affecting the peripheral neurons and is characterized by an ascending paralysis. Three clinical forms can be distinguished, the last one being the Miller Fisher syndrome, where ataxia and ophthalmoplegia

are observed. *C. jejuni* enteritis is the infection most frequently observed before Guillain Barré syndrome and occurs in 30 to 50% of all cases. It is estimated to occur in 1 in 3 000 *C. jejuni* infections. The pathogenic mechanism relies on antigen mimicry between oligosaccharides from the *C. jejuni* lipopolysaccharides and the GM_1 ganglioside of the peripheral neuron membrane [195]. The serogroup first described in Japan is *C. jejuni* PEN19 but other serogroups have been described in Europe [37]. This syndrome is very severe, leading to a 2 to 3% mortality and major neurological sequelae in 20% of the cases. The other patients experience a partial or total recovery [22]. The most severe cases are induced by *C. jejuni* [77, 183].

Recently, Helms et al. [59] in Denmark evaluated the global mortality rate of patients in the year following a bacterial enteric infection, after an adjustment on comorbidity, and surprisingly found an excess mortality after *Campylobacter* infection (OR = 1.35, 95% CI = 1.02–1.80).

In summary, *Campylobacter* infections are very common self-limited diseases. Their frequency generates numerous health care expenses. Furthermore, life-threatening systemic diseases are diagnosed more and more readily and the most severe Guillain Barré syndromes are the post infectious consequence of this infection, making *Campylobacter* infection a major public health issue.

4. HUMAN EPIDEMIOLOGY AND FOODS OF ANIMAL ORIGIN

Campylobacter jejuni is now recognised as one of the main causes of bacterial foodborne disease in many developed countries with *Campylobacter coli* less frequently implicated [43]. Foods of animal origin, in particular poultry, have been identified as significant sources of this enteropathogen as a result of infection and contamination at the pre-harvest and harvest levels [126]. The handling and consumption of poultry meat has been previously linked to human illness [8, 9], especially when eaten raw and undercooked or recontaminated following cooking.

4.1. Campylobacters and poultry

The role of poultry in the epidemiology of human Campylobacteriosis was clearly demonstrated in Belgium during the dioxin crisis in 1999. As a result detecting feeds contaminated with abnormally high levels of dioxins in feeds, domestically produced chicken and eggs were withdrawn from retail outlets in Belgium. The resulting temporary deficit in supply of poultry and eggs over subsequent months resulted in an estimated 40% reduction in the numbers of human *Campylobacter* cases reported. The incidence of *Campylobacter* cases rose to similar levels to those observed prior to the crisis when the ban on poultry meat was lifted [182].

Intestinal colonisation in broiler chicks is rarely detected until at least 7 days of age. Once colonised, chicks normally remain asymptomatic carriers until they reach slaughter age [51]. Wide variations in flock infection prevalences up to 100% have been previously reported in surveillance studies [64]. The most significant routes of transmission by *Campylobacter* to commercial poultry flocks at the pre-harvest level remain unclear. However, a number of epidemiological studies have suggested that inadequate disinfection between chick placements, age disposition, the use of multi-unit sites, the proximity of other livestock, season and lapses in biosecurity are significant risk factors [21]. The role of other vectors such as litter beetles, house flies and wild birds have also been identified as potential transmission risks [27]. Conflicting reports have emerged on the ability of campylobacters to infect successive generations either by direct vertical transmission from hen to chick via the egg or by horizontal transmission within the hatchery environment [125, 162]. Difficulties in the identification of

significant infection routes to commercial flocks at farm level have been further compounded by the strain diversity observed in both flock and environmental isolates from various studies and the frequently observed co-infection of birds with multiple strains of *Campylobacter jejuni* [108, 125]. The use of contaminated water for drinking in poultry houses has also been recognised as a significant risk factor for colonisation by *Campylobacter* and may in fact be underestimated due to the existence of viable-non-culturable or highly stressed forms of the organism in environmental samples, including farm water supplies [109].

The high prevalence of campylobacters in poultry flocks at the pre-harvest level is further exacerbated due to multiple opportunities for cross-contamination to occur during slaughter and processing. The high throughputs of modern poultry slaughter plants has necessitated the development of automated equipment in, for example, the stages of scalding, plucking and evisceration. The net effect of processing large numbers of carcases from different sources very often leads to the dissemination of enteric pathogens, including *Campylobacter* from the early stages of the slaughtering process. Also, as skin is normally not removed from dressed carcases, large numbers of campylobacters can remain in situ on the finished raw product thus increasing the likelihood of exposure to the consumer.

4.2. Campylobacters and other food animals

The gastrointestinal tracts of other food animal species have also been shown to be frequently colonised with campylobacters, particularly, *C. jejuni* and *C. coli* [99]. Reported rates of intestinal *Campylobacter* carriage in food animals have varied widely between studies [23]. The digestive tract of clinically normal cattle has been demonstrated to be a significant reservoir for a number of *Campylobacter* spp. [12], with prevalences of the enteropathogen in cattle ranging from 0–80%. Prevalences of *Campylobacter* in sheep have been shown to be generally lower with approximately 20% of animals intestinal carriers [197]. The high prevalences of campylobacters in pigs have been reported previously in numerous studies and dressed pig carcases have been shown to be more frequently contaminated than either beef or sheep [107]. This is most likely attributable to the fact that pig carcases undergo a communal scalding stage early in the slaughter process combined with the fact that the skin remains on the carcase following all of the dressing procedures.

Contaminated shellfish have also been implicated as a vehicle in the dissemination of Campylobacteriosis. Harvesting shellfish from *Campylobacter*-contaminated waters would appear to be the most likely cause of infection [193]. Campylobacters have also been isolated frequently from asymptomatic companion animals, with symptoms of enteritis frequently reported in younger animals [56]. Transmission of campylobacters from pets to humans has been confirmed in previous case studies and identified as a potential risk factor in epidemiological investigations, particularly young children in contact with puppies exhibiting enteritic symptoms [158].

4.3. Control in foods of animal origin

A longitudinally integrated approach to controlling campylobacters along the entire food chain should be adopted for foods of animal origin, in particular, poultry. Control should be directed primarily at the prevention of colonisation in food animals through the implemention of Good Hygienic Practices (GHP), biosecurity measures and husbandry practices which should be incorporated in Hazard Analysis Critical Control Point (HACCP) based risk management systems [189]. Efforts at harvest level should be concentrated on practices designed to control and reduce levels of faecal contamination during live bird transportation, slaughter and carcase dressing [9, 191]. In addition consumers and food handlers

should be made aware of the role that they play in reducing the incidence of *Campylobacter* infection by preventing cross-contamination in kitchens or food preparation areas [61].

Other potential options currently available to reduce the levels of this enteropathogen on food animal carcases include, irradiation [100], chemical decontamination [190], steam pasteurization and hot water immersion [192].

More recently, l'Agence française de sécurité sanitaire des aliments (AFSSA) has published an extensive review article entitled "*Appréciation des risques alimentaires liés aux Campylobacters: Application au couple poulet/*Campylobacter jejuni", which should be consulted for further information. This may be obtained on-line at http://www.afssa.fr.

5. ENVIRONMENTAL CAMPYLOBACTERS

5.1. Water

Waterborne outbreaks associated with contamination of drinking water by *Campylobacter jejuni* are rather common in the Nordic countries Sweden, Norway or Finland, where in sparsely populated districts groundwater is commonly used without disinfection. Campylobacters, *Escherichia coli*, or other coliforms have rarely been detected in potential sources. Using a combination of Penner serotyping and pulsed-field gel electrophoresis (digestion with SmaI and KpnI), Hanninen et al. [57] studied three waterborne outbreaks in Finland caused by *C. jejuni* and used sample volumes of 4 000 to 20 000 mL for analysis of Campylobacters and sample volumes of 1 to 5 000 mL for analysis of coliforms and *E. coli*, depending on the sampling site, confirming the likely reservoir of an outbreak. Poor water quality, sanitation and hygiene account for some 1.7 million deaths a year world-wide (3.1% of all deaths and 3.7% of all DALY's), mainly through infectious diarrhoea. Gastrointestinal diseases are often severe due to under-nutrition and lack of intervention strategies in the developing nations and virtually 9/10 account for infant deaths alone [11]. Major enteric pathogens in the infant's mortality include rotavirus, *Campylobacter jejuni*, enterotoxigenic bacteria (*Escherichia coli*, *Shigella* spp. and *Vibrio cholerae* 01) and possibly enteropathogens (*E. coli*, *Aeromonas* spp. *V. cholerae* O139) enterotoxigenic *Bacteroides fragilis*, *Clostridium difficile* and *Cryptosporidium parvum*. All except the *C. parvum* are easily controlled by chlorination of water, but re-contamination of treated water is a huge problem. Emerging environmental pathogens, such as *Helicobacter pylori* and *Burkholderia pseudomallei*, may well be of significance in some regions. In adults, much less is understood of various sequelae such as myocarditis, diabetes, reactive arthritis and cancers some months-years after initial infections. Also, besides the traditional pathogens (helminths, *Entamoeba histolytica*, *Giardia lamblia* hepatitis A and E) various enteroviruses, *C. jejuni* and *H. pylori* are emerging issues in adults.

5.2. Sewage and water treatment plants

The presence of bacterial pathogens (*Listeria monocytogenes, Campylobacter coli* and *jejuni, Escherichia coli* O157 and *Salmonella* spp.) in eight Swedish sewage treatment plants (STP), with four different treatment methods, focusing on detection of zoonotic bacteria in raw and treated sludge were investigated [142]. Restriction enzyme analysis and pulsed field gel electrophoresis of *Salmonella* serotypes indicated that *Salmonella* persists in STP and that there is a continuous supply of new strains. There are differences in treatment methods concerning the reduction of pathogens and indicator bacteria. If spread on arable land, sludge increases the environmental load of pathogens and thereby increase the risk for spreading diseases to

people and animals. Said et al. [143] reported outbreaks of infectious diseases over the last 30 years associated with private water supplies (PWS). The majority (16 outbreaks) were reported after the introduction of enhanced surveillance. Although PWS only serve 0.5% of the population, 36% of drinking water outbreaks are associated with PWS. The main pathogen, *Campylobacter*, was implicated in 13 (52%) outbreaks. Most reported outbreaks (88%) occurred in commercial or Category Two supplies, which potentially affect larger populations. The main factors implicated in these outbreaks are temporary or transient populations, treatment (lack or failure), the presence of animals and heavy rains. The public health problem associated with PWS could be prevented by the identification and understanding of risk factors, by the proper protection of water sources and adequate treatment and maintenance. This could be facilitated through the introduction of a risk assessment as part of a scheme for the water supplies. Ottoson [120] investigated the prevalence of pathogens (e.g. rotavirus, *Salmonella typhimurium*, *Campylobacter jejuni*, *Giardia lamblia* and *Cryptosporidium parvum*) in greywater in a local treatment system at Vibyasen (north of Stockholm, Sweden) and the faecal load of these pathogens and were used to form the basis of a screening-level quantitative microbial risk assessment (QMRA) using faecal indicator bacteria and chemical biomarkers. Growth conditions for *Salmonella* in greywater sediments were also investigated and risk modelling based on replication in the system increased the probability of infection from *Salmonella* 1000-fold, but it was still lower than the risk of a rotavirus infection. The microbial quality of several, usually untreated, surface domestic water sources, used by rural communities in the Venda Region of South Africa, was assessed to gauge their fitness for human consumption and to highlight the possible impact of waterborne diseases. *Salmonella, Shigella*, *Vibrio cholerae*, *Campylobacter*, *Aeromonas* and *Plesiomonas* were isolated from several of the water sources investigated. The use of these water sources for drinking and domestic purposes poses a serious threat to the health and well-being of the users and calls for urgent South African government intervention [112].

5.3. Farms

Campylobacter is the most commonly reported notifiable disease in New Zealand. Savill et al. [150] investigated the reservoirs of *Campylobacter* in a defined geographical area within New Zealand and compared strains isolated from humans and environmental sources within this area as a prelude to investigating the likely transmission routes to humans. *Campylobacter jejuni* was commonly found in faeces from dairy cows, beef cattle, sheep and ducks, chicken carcasses, sheep offal and surface waters and *C. coli* was commonly found in sheep faeces. Minihan et al. [99] reported that the prevalence of *Campylobacter* spp. faecal shedding within pens was positively correlated to the pen, the month of sampling and the *Campylobacter* spp. contamination status of the pen dividing bars and the water trough surface. They suggested that *Campylobacter* spp. should be considered as a pathogen shed in the faeces of a substantial proportion of feedlot cattle. Guan and Holley [55] reviewed available international data and the developing situation in Western Canada upon the survival of major pathogens including *Escherichia coli* O157:H7, *Salmonella*, and found significant variability in resistance to environmental challenge that are characteristic of the organisms themselves. The survival of pathogens were longer in environmental samples at cool temperatures but their abilities differed when exposed in liquid and solid manure. Theoretical extrapolations from cattle manure environments, indicated that holding manure at 25 °C for 90 days would appear to render the cattle manure pathogen-free. However, with good hygienic practice during harvest, a very low level of this pathogen can be achieved on dressed carcasses. Poultry,

particularly chickens, account for the majority of human infections caused by *Campylobacter*. Reduction or elimination of this pathogen in the poultry reservoir is an essential step in minimizing the public health problem. However, farm-based intervention measures are still not available because of the lack of understanding of the ecological aspects of *C. jejuni* on poultry farms and Sahin et al. [141] have elaborately discussed the horizontal and vertical transmissions of *Campylobacter* infections affected by immune status of the poultry host and the environmental conditions in the production system. Eifert et al. [36] compared various sampling techniques (cloacal swabs, faecal samples and environmental surface "drag" swabs) on 3, 5 and 7 weeks old poultry birds for presence of *Arcobacter butzleri*, a causal agent of human enteritis and found that environmental swabs recorded the highest percentage recovery, while intestinal tracts had none. Gaynor et al. [47] demonstrated via gene expression studies the capacity of *C. jejuni* to adapt to multiple environmental niches. The genetic evolutionary mechanisms of adaption provides the first whole-genome molecular exploration of the effect of laboratory culture and storage on colonization and virulence properties of this pathogen. In this respect, it is interesting to note that *Campylobacter* cases occurring in rural populations of Michigan, USA, attributable to poultry husbandry and of some cases occurred in individuals who were not poultry farmers by occupation, is highlighted by the studies of Potter et al. [131].

5.4. Food related environments

Novel employment of lactate dehydrogenase release from porcine aortic endothelial cells (PAEC) as a quantitative marker of cytotoxic activity in thermophilic *Campylobacter* spp. including *Campylobacter jejuni, C. coli, C. lari* and urease-positive thermophilic campylobacters (UPTC), from human faecal isolates, poultry and environmental sources has been demonstrated by Millar et al. [98]. Enterobacteriaceae and *Campylobacter jejuni*, were determined by quantitative real-time PCR (qPCR) than by cultivation [73], as some of these bacteria may have been in a potentially hazardous active but non-cultivable state and this method provides a viable alternative for biosafety and hygiene monitoring reasons. Yang et al. [194] reported that retail chicken meat, raw milk and environmental water are commonly contaminated with *C. jejuni* and could serve as a potential risk for consumers in eastern China, especially if proper hygienic and cooking conditions are not maintained. The rapid and sensitive detection of *C. jejuni* is necessary for the maintenance of a safe food/water supply in China and the real-time PCR assay provides a specific, sensitive and rapid method for quantitative detection of *C. jejuni*. Pearce et al. [124] reported that although *Campylobacter* is highly prevalent in the intestinal tracts of swine arriving at the slaughter facility, this microorganism does not progress through the slaughtering operation and is not detectable on carcasses after overnight chilling. Sharma et al. [152] critically examined the potential of emerging water-borne pathogens in both developed and developing nations and the global epidemiology of a number of cases involving hepatitis viruses (including hepatitis E virus), *Campylobacter jejuni*, microsporidia, cyclospora, *Yersinia enterocolitica*, calciviruses and environmental bacteria like *Mycobacterium* spp., Aeromonads, *Legionella pneumophila* and multidrug-resistant *Pseudomonas aeruginosa* that have been associated with water-borne illnesses. It also examines the possible reasons, such as an increase in the number of immunocompromised individuals, urbanization and horizontal gene transfer, that may underlie their emergence. The isothermal amplification method nucleic acid sequence-based amplification (NASBA), which amplifies RNA, has been reported as useful for the detection of microbial pathogens in food and environmental samples [29].

More recently Birk et al. [18] have developed a food-based model system which is

a suitable model system for the study of survival of *C. jejuni* in food systems. This model employs chicken juice as the test matrix and may be useful in anticipating the survival of *C. jejuni* in foods, thereby leading to the development of new preservation systems.

6. ATYPICAL CAMPYLOBACTERS

In relation to human Campylobacteriosis, *C. jejuni, C. coli, C. upsaliensis, C. hyointestinalis, C. lari, C. fetus, C. sputorum* biovar sputorum have been demonstrated to be implicated as gastrointestinal pathogens, though some are rare [71]. *C. concisus, C. curvus, C. gracilis, C. rectus* and *C. showae* are detected in association with the oral cavity [82]. Alternatively, *C. mucosalis, C. helveticus* and *C. sputorum* biovar faecalis are isolated from animals [114, 161].

Moreover, some other atypical and emerging *Campylobacter* organisms than those described above have interestingly been identified to occur for these ten years. Therefore, the aim of the present article is to review atypical and emerging examples among the genus. Five years after, following the original description of *C. lari* organisms by Skirrow and Benjamin in 1980 [155], Bolton et al. [20] isolated the first 10 atypical isolates of *C. lari*, urease-positive thermophilic *Campylobacter* (UPTC) from the natural environment in England in 1985 [20]. This was the first example of urease-producing bacteria among the genus *Campylobacter*. Then, four UPTC isolates were found for the first time from humans, two from the faeces with two diarrheal diseases, one from the appendix with one appendicitis and one from the urine with one urinary tract infection in 1986–1989 [17, 96]. Until now, only these four clinical cases of UPTC isolates have been published. However, any association of UPTC with human disease still remains unclear. UPTC organisms were demonstrated to belong within *C. lari p*ossibly as a biovar [122] or a variant [96]. After these descriptions of UPTC appeared, isolates of UPTC have been reported in several European countries (The Netherlands in 1997 [39], Northern Ireland in 1996, 1999 and 2003 [66, 93, 193], England in 1998 [42]) and one Asian country (Japan in 1996 and 2002 [92, 94]). Consequently, about 200 UPTC isolates have been found from the natural environment, river water, sea water and shellfish, including wild birds, but not from any domestic or wild animals. Therefore, the natural environment is an important reservoir of the UPTC organisms. However, in a study by Waldenstrom et al. [184] were unable to detect any UPTC organisms in a wild bird population in Sweden.

When On and Harrington studied the taxonomic and epidemiological relationship among *Campylobacter* species by numerical analysis of amplified fragment length polymorphism (AFLP), a high level of genetic diversity in *C. lari*, particularly amongst UPTC isolates, was identified [117]. Multilocus enzyme electrophoresis analysis, which were shown to be the most successful at discriminating UPTC organisms at the subspecies level, whereas serotyping, phage-typing, antibiogram typing and flagellin typing were unsuccessful [101], also demonstrated that the UPTC isolates ($n = 31$) isolated from several countries and sources examined are genetically hypervariable and form a cluster separate from the *C. lari* ($n = 3$) cluster [94].

In relation to the pathogenesis, Sekizuka et al. [151] has found short *fla*A-like sequences, containing internal termination codons (TAG), incomplete genes or pseudogenes of *fla*A in two Japanese UPTC isolates [151]. Furthermore, shorter *fla*A genes without any internal termination codons than those of *C. jejuni* and *C. coli* were demonstrated in the isolates of UPTC from the natural environment in England [151] and in Northern Ireland (T. Gondo, unpublished data). The reason(s) why any of UPTC organisms have not been identified as a cause of gastrointestinal disease for humans, may partly be due to the shorter and/or

shorter pseudogene structure of *flaA*. No phenotypic and genotypic characteristics of urease from UPTC have yet been described.

In 1983–1985, *C. hyointestinalis* organisms, distinguished from previously described catalase-positive *Campylobacter* species by colony morphology, ability to produce H_2S in triple sugar iron agar, glycine tolerant, intolerant to 3.0% NaCl, ability to grow 25 °C, sensitive to cephalothin and resistant to nalidixic acid, were first isolated from the intestines of pigs with proliferative enteritis and other animals (faeces from cattle and intestine of a hamster) [49, 50]. About ten years after from the first description of *C. intestinalis*, seven isolates resembled but distinct from the type strain and other reference strains of *C. intestinalis* were obtained from porcine stomachs [116]. Based on the numerical analysis of 38 phenotypic characters, DNA-DNA hybridization studies and DNA base compositions, On et al. [116] proposed the name *C. hyointestinalis* subsp. *lawsonii*. Alternatively, *C. hyointestinalis* subsp. hyointestinalis was accordingly given [116]. When Harrington and On examined phylogenetic relationships of *C. hyointestinalis* subspecies by means of 16S rDNA sequences, they found that the sequence identities among *C. hyointestinalis* subsp. *lawsonii* isolates exceeded 99.9% and among *C. intestinalis* subsp. *hyointestinalis* isolates ranged from 96.4 to 100% [58]. Sequence identities between isolates representing the two subspecies ranged from 95.7 to 99.0%. Surprisingly, an interventing sequence was identified in the *C. hyointestinalis* subsp. *lawsonii* strains [58]. AFLP fingerprinting method was also demonstrated to allow the classification of the *C. hyointestinalis* at the subspecies level [35].

When, in 1998, Lawson et al. [70] examined saliva and faeces from 20 healthy individuals in order to know the variety of campylobacters in their gastrointestinal tract, PCR assays specific for nine species of *Campylobacter* (*C. sputorum, C. concisus, C. upsaliensis, C. helveticus, C. lari, C. fetus, C. hyointestinalis, C. jejuni and C. coli*), and for the genus as a whole was performed [70]. Three unidentified 16S rDNA *Campylobacter* genus-specific amplicons of faecal origin were sequenced and demonstrated to be 99% similar [70]. These were previously undescribed and uncultivated *Campylobacter* species. The organism from faeces, specific PCR assay, was detected in 10 of the 20 faecal samples but not in any saliva samples. Then, the authors proposed to term "Candidatus *Campylobacter hominis*" [70]. Nextly, they developed an isolation strategy employing initial non-selective membrane filtration onto fastidious anaerobe agar for the uncultivated *C. hominis* organisms [72]. The unique species status of the isolates, whose nearest phylogenetic neighbours were *C. gracilis* and *C. sputorum*, was further confirmed by taxonomic study of 47 phenotypic characteristics [72].

C. lanienae is a new catalase-positive species that was first described from the faeces of healthy abattoir workers in Switzerland in 2000 [81]. Nucleotide sequence of the 16S rDNA, DNA-DNA homology test and G+C content of genome DNA demonstrated that the new organism constituted a previously undescribed species, whose nearest phylogenetic neighbours were *C. hyointestinalis* subsp. hyointestinalis, *C. fetus* and *C. mucosalis* [81]. Rapid PCR-biprobe identification scheme based on the real-time PCR was developed for the *Campylobacter* taxa pathogenic for humans, including *C. lanienae* [82]. This new organism has also been isolated from the faeces of six healthy pigs in Japan [148] and from the faeces of bovine and beef cattle, in the beef cattle, *C. lanienae* was the most frequently detected species (49%), in Canada [62, 63, 115, 118]. In addition, an intervening sequence of 226 bp in the 16S rDNA was found in four isolates of the six of *C. lanienae* in Japan [148].

In 1998, On et al. first identified the 15 strains isolated from faeces of 14 cattle in United Kingdom and one human diarrhoea in Canada among 44 catalase negative and urease-positive *Campylobacter*

group as a new *C. sputorum* biovar paraureolyticus, by a phylogenetic study based on phenotypic characterization, numerical analysis of whole-cell protein profiles, DNA-DNA hybridization and sequence analysis of 16S rDNA [115]. They demonstrated the clonality of *C. sputorum* bv. paraureolytics determined by macrorestricion profiling and biotyping by using the 18 isolates isolated over a 12-month period from seven dairy cows contained in a single herd [181], Their study also indicated that the organism can persist in cattle for a long-term, at least 12 months.

Alderton et al. in 1995 [5] reported the 11 isolates from intestinal lesions of pigs with proliferative enteritis including an organism formerly described as strain RMIT $32A^T$ as a new name *C. hyoilei* sp. nov. [33]. The phenotypic characteristics of these organisms indicated that they are closely related to each other and are not isolates of other *Campylobacter* spp. commonly isolated from pigs. They also suggested that this organism is more closely related to *C. jejuni* than *C. coli* based on the sequence differences of 16S rDNA. However, it was confirmed that *C. coli* strains and *C. hyoilei* strains were indistinguishable based on examining a variety of phenotypic and genotypic criteria and both represent the same species [180]. Although differentiation between *C. hyoilei* and *C. coli* using genotypic and phenotypic analyses were demonstrated, the taxonomic subcommittee of the International Committee on Systematic Prokaryotes finally concluded that the epithet 'hyoilei' could in principle be revised as an infrasubspecific designation. Therefore, the subcommittee discouraged the use of the name *C. hyoilei*.

In conclusion, for the last ten years, about 200 UPTC isolates have found only in the natural environment in Europe and Japan, whereas several atypical and emerging *Campylobacter* taxons (*C. hominis, C. lanienae* and *C. sputorum* subsp. paraureolyticus) have been newly found mainly in the faeces of healthy humans and domestic animals (pigs, bovines and cattle) in Europe and North America (Canada), Moreover, seven isolates of *C. hyointestinalis* subsp. lawsonii have been isolated from the porcine stomach in UK. Thus, healthy domestic animals including wild animals could potentially be important reservoirs of these new atypical and emerging organisms of *Campylobacter* in humans.

7. ANTIBIOTIC RESISTANCE

Campylobacter enteritis is considered to be a zoonotic disease, and domestic animals such as poultry, cattle and pigs can act as sources of infection [59, 95]. Transmission to man usually results in sporadic infection, and is often associated with improper handling or cooking of food. The majority of cases of clinical *Campylobacter* enteritis are sufficiently mild or self-limiting not to require antimicrobial chemotherapy [6]. Nevertheless, in severe or recurrent cases where antibiotics are required, susceptibility testing is important to ensure appropriate and timely treatment [13, 134, 179]. Serious systemic infection may also be treated with an aminoglycoside such as gentamicin [153]. Tetracyclines have been suggested as an alternative choice in the treatment of clinical *Campylobacter* enteritis, but in practice are rarely used. However, macrolides remain the agents of choice, and resistance rates to erythromycin remain comparatively low [104]. Fluoroquinolones, offer an effective therapy, against most enteric pathogens, to treat acute bacterial diarrhoea; with ciprofloxacin being used extensively as prophylaxis for travellers [52]. Emergence of resistance to these agents however, has since made their efficacy less certain. Resistance was reported to develop among patients after treatment with fluoroquinolones [14], and was also found to coincide with the introduction of these agents in veterinary medicine [1, 2, 38]. However, an increasing number of *Campylobacter* isolates resistant to these drugs are now being cultured from both

clinical and food samples in several European countries, Canada and the United States. Since the 1990s, a significant increase in the prevalence of resistance to macrolides and fluoroquinolones among *Campylobacter* spp. have been reported and this is recognised as an emerging public health problem in many European countries [40]. Entry of these isolates into the food chain could represent a significant threat to public health.

7.1. Antimicrobial susceptibility testing in *Campylobacter* spp.

Several laboratory methods, including disc diffusion, broth microdilution, agar dilution and the Epsilometer-test (E-test) have been applied to determine in vitro susceptibility profile(s) of *Campylobacter* to a range of antimicrobial agents [15, 41, 45, 46, 48, 84, 119, 149]. Despite the availability of comparable standardised procedures for many organisms, based on, the approved guidelines defined by the National Committee for Clinical Laboratory Standards (NCCLS), no internationally accepted criteria for susceptibility testing of *Campylobacter* spp. are available and breakpoints do not exist. Consequently it is not possible to directly compare the resistance profiles of isolates cultured from various origins. More, recently however, the NCCLS Sub-Committee on Veterinary Antimicrobial Susceptibility Testing approved an agar dilution protocol as a valid method.

Several authors have compared the performance of the methods above and reported a correlation between E-test and agar dilution methods. Values determined however can vary depending on the antimicrobial agent(s) being considered [48]. This observation was particularly evident with respect to *C. jejuni* [84]. Comparing MIC values obtained by E-test and the agar dilution protocols, Ge et al. [48] reported values ranging from 21.4 to 62% for gentamicin and nalidixic acid respectively. Whilst the E-test is convenient, relatively simple to perform, MIC values determined by this method are lower when compared to similar values obtained by the agar dilution method regardless of the organism tested [41, 48, 60]. However when E-test and the agar dilution method are used on a small number of isolates from a single geographic location, acceptable agreement between both approaches for susceptibility categorisation is achieved. For larger collections, microdilution is the preferred protocol especially when susceptibility to nalidixic acid and trimethoprim-sulfamethoxazole are being considered [84].

Molecular techniques offer an alternative means of assessing antimicrobial resistance among bacterial isolates. In a study of quinolone-resistant *Campylobacter* a majority of isolates analysed were shown to possess a common mutation [128]. The predominant genetic alteration responsible for conferring resistance to ciprofloxacin in *C. jejuni* and *C. coli* is the result of a mutation in the *gyrA* gene, whereby many isolates tested demonstrated a Thr-86-Ile substitution in the A-subunit of DNA gyrase [185]. A Mismatch Amplification Mutation Assay (MAMA)-PCR has been successfully applied to the detection of ciprofloxacin resistance in *C. jejuni* and *C. coli*, and this protocol is a convenient screening tool among these isolates [196]. This method used a conserved forward primer and a reverse diagnostic primer, which together generate a 264-bp product that is a positive indication of the presence of the Thr-86-Ile amino acid substitution, consistent with resistance to ciprofloxacin. A "real-time" PCR-based approach was recently developed to detect the C-to-T nucleotide polymorphism associated with the latter amino acid substitution in the *gyrA*-encoding gene [34]. In this case, fluorescence resonance energy transfer technology (FRET) can be applied to the analysis of melting curves when a specific probe hybridises to a DNA template. This protocol can be adapted to include additional mutations providing rapid and reproducible screening methods for ciprofloxacin resistant *Campylobacter* isolates.

Undoubtedly, one of the advantages of using these methods includes the possibility of direct detection from a sample obviating the need for culture [28]. Molecular methods can facilitate analysis of organisms that may be sub-lethally damaged and difficult to grow, and these strategies also offer the possibility of screening large numbers of isolates for a specific mutation within a single assay. The disadvantages of using molecular detection methods include the failure to detect resistance if a new, unexpected or rare resistance mechanism is present [104], and the necessity to perform a separate assay for each antimicrobial agent tested. Furthermore, no standards exist for performing genetic testing methods [28]. For these reasons, it may be more useful to combine phenotypic and genotypic methods of susceptibility testing.

7.2. Surveillance of antimicrobial resistance in *Campylobacter* spp.

Transmission of antimicrobial resistance from food animals to humans can occur via the food chain [126, 133]. It is difficult to determine the precise extend of the risk posed to human health [127]. Nevertheless, food animals are a significant reservoir of antibiotic resistant zoonotic pathogens. Continuous monitoring of susceptibility profiles of *Campylobacter* spp. to a panel of antimicrobial agents is necessary for a number of reasons. Firstly, there are increasing rates of resistance to the agents of choice used in the treatment of clinical enteric infection [1]. This suggests a need to supply alternative antimicrobials which remain therapeutically effective. Secondly, the emergence of multidrug-resistant (MDR) organisms must be monitored carefully [86]. Finally, mechanisms for the transfer of resistance both within *Campylobacter* spp. and between different genera of enteric organisms by means of mobile genetic elements may present a significant threat to the continued efficacy of antimicrobial chemotherapy [76, 113].

The use of antimicrobial agents on farm animals, both to treat infection and as growth promoters is a cause of concern, and the increasing rates of resistance among *Campylobacter* spp. to these agents appear to make a conservative policy on the use of antibiotics in farm animals advisable [1, 127]. Antibiotics of the macrolide-lincosamide group have been used in treating food animals worldwide for several decades. Their uses have included the control of dysentery and *Mycoplasma* infections in swine, and for treating mastitis in cattle [40]. The use of macrolides and other compounds for growth promotion has been banned, in all European Union countries with effect from July 1999. Fluoroquinolones are available for treating food animals in many countries, and Table I shows the veterinary licensing "time-line" of this group of antibiotics in a number of European countries. It is difficult to evaluate the actual usage of these agents in food animals, but it is noteworthy that fluoroquinolone treatment of *Campylobacter*-colonised broiler chickens has induced fluoroquinolone resistance under experimental conditions [65].

Supplementing animal feed with antibiotics is estimated to constitute more than half the total antimicrobial use worldwide [188]. It has been reported that in Denmark the consumption (per animal) of antibiotics such as macrolides and tetracyclines in agriculture was 2–4 times higher than consumption (per patient) in human medicine [1]. Emergence of antimicrobial resistance among zoonotic pathogens has led to the development of a continuous surveillance system of antimicrobial resistance among bacteria isolated from pigs, cattle and broilers in Denmark. The Danish Integrated Antimicrobial Monitoring Programme (DANMAP) has set out to establish a baseline for comparison with future prospective studies to enable the determination of trends over time [1]. Monitoring strategies such as this may have a positive impact on the effective treatment of human enteric *Campylobacter* infection. To date, with the exception of ciprofloxacin resistance, there is a scarcity

Table I. Veterinary licensing of fluoroquinolones in selected European countries.

Country	Antimicrobial	Licensing year	Animal species
Ireland	Enrofloxacin	Prior to 1987	Cattle, pigs, poultry
United Kingdom	Enrofloxacin	1993	Cattle, pigs, poultry
	Danofloxacin	1993	Poultry
	Marbofloxacin	1995	Cattle
	Difloxacin	1998	Poultry
Denmark	Enrofloxacin	1991	Cattle, pigs, poultry
	Danofloxacin	1993	Poultry
	Difloxacin	1998	Poultry, turkey
	Marbofloxacin	1998	Cattle, pigs, dogs, cats
Spain	Enrofloxacin	1986	Cattle, pigs, poultry
	Difloxacin	1998	Poultry
The Netherlands	Enrofloxacin	1987	Cattle, pigs, poultry
	Difloxacin	1998	Poultry
France	Enrofloxacin	1991	Cattle, poultry
	Danofloxacin	1996	Cattle
	Marbofloxacin	1993	Cattle
	Difloxacin	1998	Poultry

of scientific evidence for the transmission of antimicrobial resistance as a direct result of the use of antimicrobial agents in veterinary medicine [127].

7.3. Genetic mechanisms associated with antimicrobial resistance in *Campylobacter* spp.

Bacterial populations can respond to the threat of an antimicrobial agent by evolving some type of resistance mechanism(s) [138, 159]. The imposed selective pressure results in the development of a corresponding resistance determinant, either through direct acquistition or intrinsically by modification of a host gene target, designed to facilitate evasion of the inhibitory substance. For example environmental selection following enrofloxacin treatment of chickens infected with fluoroquinolone-sensitive *Campylobacter* spp. resulted in the emergence of the corresponding resistant isogenic strains suggesting that this organism is hypermutatable under these conditions [87]. Horizontal transfer of such resistance determinants (acquisition) together with any genetic modification of pre-existing genes through point mutations (intrinsic) or some other genetic event, are thought to be the main mechanisms contributing to bacterial resistance [2, 10, 140]. Self-transmissible elements including plasmids, transposons and bacteriophage all facilitate the acquisition and subsequent dissemination of resistance determinants. In addition, integrons, when associated with plasmids and/or bacteriophage are now considered efficient vehicles for the transfer of resistance markers among unrelated bacterial populations [24].

The isolation rate of plasmids from *Campylobacter* spp. has been shown to vary considerably between 44 and 91% for clinical and poultry isolates respectively in one study [75], compared with a plasmid isolation rate of 19% reported in a separate study [14]. As shown in Table II, tetracycline, kanamycin and chloramphenicol resistances are primarily plasmid-mediated. Historically, tetracycline resistance has been

Table II. Genetic mechanisms responsible for antimicrobial resistance detected to date in *C. jejuni* and *C. coli*.

Antibiotic	Mechanism of resistance	Reference
Aminoglycosides (with the exception of kanamycin)	Chromosomal: enzymatic modification of antibiotics. Integron-mediated resistance	[113, 175]
Kanamycin	Majority plasmid-borne, remainder chromosomal; resistance through enzymatic modification of kanamycin	[140]
Chloramphenicol	Plasmid-borne, resistance through modification of the target site (ribosome) or alteration of the antibiotic	[186]
Ciprofloxacin	Chromosomal: modification of *gyrA* and *parC* confers resistance	[3, 4, 16]
Erythromycin	Chromosomally mediated, resistance through modification of the target site (ribosome)	[166]
β-Lactams	Chromosomal; three mechanisms, decreased uptake through modification of a porin, alteration of a penicillin binding protein, or production of a β-lactamase	[129]
Tetracycline	*tetO* gene, plasmid-borne in the majority of cases, resistance mediated through ribosomal protection	[7, 78, 167]
Trimethoprim	*dfr1* gene, chromosomal, located to the remnants of an integron *dfr9* gene, chromosomal, located to the remnants of a transposon Resistance arising through modification of the trimethoprim target	[53]
Multidrug-resistance (MDR)	Efflux pump with a broad specificity; preventing accumulation of antibiotics	[25]

particularly well researched and documented [173] The *tetO* gene conferring tetracycline resistance has a G+C content of 40% [90], which is close to that of the *tetM* gene of *Streptococcus pneumoniae*, with which it shares 75% homology [89]. It is significantly higher than that for *C. jejuni* and *C. coli* chromosomal (32.5 mol%) and plasmid (33 mol%) DNAs [90, 169]. Based on this evidence, Taylor and Courvalin [168] have suggested that the *tetO* gene was acquired by *Campylobacter* spp. from a Gram-positive coccus, and that divergence occurred over time. More recently, in Brazil, only 15.9% of the isolates analysed for plasmids contained these mobile elements and none of the tetracycline resistant strains were found to harbour plasmid DNA [10].

Chloramphenicol resistance, although rare in campylobacters [10], has also been reported to be plasmid mediated [140]. A chloramphenicol resistance determinant cloned from a *C. coli* plasmid was sequenced and found to have 43 and 57% homology with chloramphenicol acetyltransferase (CAT) proteins from other Gram-positive and -negative origins [186]. A kanamycin resistance determinant, *aphA*-3 was found located distal to

this *cat* gene and Sagara et al. [140] reported a link between kanamycin and chloramphenicol resistance in *Campylobacter* following a number of cloning experiments involving the *cat* and *kan* resistance genes of a plasmid from a multiple-antibiotic-resistant *C. coli* isolate.

Kanamycin resistance in *Campylobacter* is more commonly associated with *C. coli* than with *C. jejuni* [140]. Like tetracycline resistance, kanamycin resistance determinants were located both on the chromosome and on self-transmissible plasmids [168]. These determinants are frequently found on the same plasmids as tetracycline-resistance determinants [67, 170]. Kotarski et al. [67] also observed that the Km^r determinant could translocate between plasmid and chromosomal DNA, suggesting that the Km^r determinant in campylobacters may be located on a transposable element of approximately 4 kb. A number of genes responsible for Km^r in campylobacters have been identified including *aphA*-1, *aphA*-2, *aphA*-3 and *aphA*-7, with a plasmid location reported for both *aphA*-3 and *aphA*-7 [168, 172]. The *aphA*-3 gene is often found on large plasmids that also encode *tetO*. *aphA*-3 like *tetO* is thought to have originated from a Gram-positive source and is commonly found in staphylococcal and streptococcal species [68]. *aphA*-7 determinants on the other hand have been reported to be found on small plasmids that do not encode any other resistance determinants [171]. Although *aphA*-7 has been shown to have a broad host range, in that it can be expressed in both *E. coli* and *S. gordonii*, its low G+C ratio (32.8%), matching that of *C. jejuni*, suggests this gene may be indigenous to campylobacters [172]. A chromosomal location for *aphA*-1 has been reported for a *Campylobacter*-like organism [121]. Table II lists some of the potential means by which *Campylobacter* acquires antimicrobial resistance markers.

Macrolides are the agents of choice for treating *Campylobacter* infections. Resistance to erythromycin is mainly found in strains of animal origin, especially *C. coli* in pigs and from chickens [40] and detection of erythromycin resistance may be determined by PCR methods [163]. Nevertheless, erythromycin is considered to be one of the safest drugs effective against *Campylobacter*. Resistance may also develop during the course of human treatment. Similar to tetracycline (outlined above), erythromycin is a potent inhibitor of protein synthesis, binding reversibly to several ribosomal targets including the Domain V-located on the 23S rRNA gene locus, in addition to the ribosomal structural proteins, L2, L4, L15 and L22. Three major point mutations occuring within the former locus, and responsible for erythromycin resistance, were defined [177]. The MIC in each case were 128 μg/mL, as defined by the agar dilution method. A combined PCR-RFLP assay was evaluated as a direct means of detection. Similarly, a PCR-based line-probe assay focusing on the 23S rRNA target only, was developed as a simple means to detect isolates resistant to erythromycin [111]. Whilst useful, this approach detected only 50% of mutations arising in resistant Japanese isolates analysed. It is consieveable that not all of the mechanisms contributing to macrolide resistance in *Campylobacter* have been described to date. The possibility arises that at least some of these will be MIC-dependent. Vacher et al. [177] did not consider the possible involvement of the ribosomal structural proteins, whereas Corcoran and Fanning (unpublished) sequenced several L4 and L22 genes and defined associated polymorphisms in resistant isolates. None of these isolates possesed any of the corresponding nucleotide polymorphisms in the 23S rRNA gene. Similarly, the involvement of an efflux system (below) cannot be ruled out [88]. Inhibition of this pump system with phenylalanine-arginene b-naphthylamide (PAβN) was shown to restore susceptibility to *Campylobacter* in a dose dependent manner.

Fluoroquinolones are important drugs used in human and animal medicine, and are often the agents of choice used to treat campylobacteriosis in humans. Currently,

in the European Union, fluoroquinolones are licenced for use in a number of food animals, however emerging resistance to this important class of antimicrobial is recognised as a significant public health problem [128]. This observation may in turn, lead to *Campylobacter*-associated deaths among vulnerable members in the community. Resistance to fluoroquinolones arises following a point mutation [A***C***A⌈A***T***A], which produces a Thr-86-Ile amino acid substitution in the Quinolone Resistance Determining Region (QRDR) of the *gyrA* subunit-encoding gene [185]. Genetic alterations in this region are often associated with high-level resistance to nalidixic acid (MIC > 64–128 μg/mL) and ciprofloxacin (MIC > 16–64 μg/mL). Similarly, resistance to fluoroquinolnes may also be associated with the activation of a multi-gene efflux pump system (see below and [25]).

The presence of chromosomally-located transposons among *Campylobacter* spp. had not been reported prior to 1998. The *dfr9* gene-encoding trimethoprim resistance was located to the remnant of a transposon inserted in the genome of a number of clinical *C. jejuni* isolates, [53]. The G+C content of *dfr9* was found to be 40%, (similar to the *tetO* gene described earlier), which is considerably higher than that of *Campylobacter* spp, and was previously detected in a transposon located in the genome of porcine isolates of *E. coli* [26]. A study by Richardson and Park [136] identified an insertion sequence that was flanked by direct repeat sequences in the chromosome of certain isolates of *C. jejuni*, which appeared to be a non-functional transposable element and the spread of antibiotic resistance under natural conditions imay be due to a combination of gene transfer systems acting in parallel or in series [30].

7.4. Gene cassettes and class 1 integrons in *Campylobacter* spp.

Integron structures are naturally occurring gene expression systems that can potentially capture and integrate one or more gene cassettes and convert them into functionally expressed genes [135]. It is these gene cassettes that encode the resistance determinants to several antimicrobial agents [24].

Although several classes of integrons have been described to date, class 1 are clinically significant. A typical class 1 integron includes two conserved segments (CS), known as the 5'- and 3'-CS segments, flanking the central gene cassette. An *intI* gene encoding an integrase enzyme is located within the 5'-CS, which is responsible for the recombination of an incoming gene cassette at a specific *att1* attachment site. Also, within this region is a promoter which facilitates the efficient expression of any integrated gene cassette. The 3'-CS contains two open reading frames (ORFs) encoding resistance to quaternary ammonium compounds (*qac*) and sulphonamide (*sul1*), respectively. Integrons can incorporate and express more than one gene cassette, provided that its location is flanked by the 5'- and 3'-CS domains. Thus integrons may contain a number of recombined gene cassettes, oriented in a classical "head-to-tail" arrangement, conferring a multi-drug resistant (MDR) phenotype on any isolate in which these genetic elements are located.

Previously, integron-like structures were reported in *Campylobacter* isolates raising the possibility that these elements may encode antimicrobial resistance and possibly function as a potential vehicle for dissemination of resistance among *Campylobacter* spp. [85]. Gibreel and Sköld [53], reported the existence of chromosomally located integrons carrying a *dfr1* containing gene cassette (Tab. II) in *C. jejuni*. In a recent study investigating of a large collection of unrelated *Campylobacter* spp., isolates of both human and animal origin, complete class 1 integrons were identified [113]. In this case, the gene cassettes contained two ORFs, one of which conferred resistance to the aminoglycoside antibiotics, streptomycin/spectinomycin.

As the use of aminoglycoside therapy may be considered as an appropriate treatment option for some *Campylobacter*-related infections, the recent identification of integrons containing aminoglycoside-encoding genes (*aadA2* and *aac4*), suggests that the possibility now exists for treatment failure to occur, due to the presence of these genetic elements in *Campylobacter* spp. [76, 113]. Furthermore, the presence of class 1 integrons in several *Campylobacter* isolates may in part offer an explanation for the high levels of resistance to sulphonamides, frequently reported among these organisms. Increasing prevalence of macrolide- and quinolone-resistance is more usually attributed to specific mutations in chromosomally located genes, though the future involvement of plasmid encoded integrons cannot be ruled out. O'Halloran et al. [113] suggest that integrons may be partly responsible for horizontal gene transfer as a potential vehicle for dissemination of MDR phenotypes among *Campylobacter* spp. These findings may have further implications for future therapeutic strategies, leading to reduced drug efficacy and/or treatment failures in the case of MDR organisms, whose transmission through the food chain poses a real threat to public health.

7.5. MDR-mediated by antimicrobial efflux systems

Active efflux pumps are known to contribute to intrinsic and acquired resistance to a range of antimicrobial agents [79, 87, 91]. These pumps reduce the intracellular accumilation of antimicrobial agents and other compounds and this feature is now recognised as a major mechanism of resistance in pathogenic organisms [110, 130, 178]. Comparitive genomics has identified a number of efflux transporters and some of these are classified as H^+-antiporters. In *Campylobacter* spp., the resistance to nodulation and cell division (RND) family [176] is associated with high-level fluoroquinolone resistance [79, 132, 187]. This resistance is linked to the activation of the *Campylobacter*-mediated efflux system referred to as the *cmeABC*-operon. This three gene operon efflux pump system contributes to multidrug resistance (MDR) in *C. jejuni* and probably in *C. coli* also (Corcoran and Fanning, unpublished) and consists of an inner-membrane transporter (encoded by the *cmeC* gene), a periplasmic fusion protein (*cmeB*) and an outer-membrane channel protein (*cmeA*). Susceptibility studies by Lin et al. [79, 80] using wild type and isogenic mutants in *C. jejuni* demonstrated that inactivation of the CmeABC pump by insertional mutagenesis substantially increased the susceptibility of *C. jejuni* to several classes of antimicrobial agent(s) and also to heavy metals and bile salts [79]. Resistance to bile salts may be a necessary step for successful colonization of animal intestines, contributing to bacterial pathogensis [87].

Efflux mechanisms have an important impact on antimicrobial resistance [187]. Resistance by efflux can be easily disseminated [91]. In several cases the genetic elements encoding efflux pumps and their regulators are located on plasmids, or on conjugative or transformable transposons located on plasmids or in the chromosome. More importantly, efflux mediated resistance mechanisms can spread between phylogenically very different species. This has been exemplified by the macrolide-mediated efflux, not only among streptococci, but also to other Gram-negative bacteria [83]. Co-transfer with genes for other resistance classes may also take place if these are present together on large mobile genetic elements.

In conclusion, thermophilic *Campylobacter* spp. are among the commonest bacterial cause of gastroenteritis in developed countries. Our knowledge of this organisms epidemiology is limited. Campylobacteriosis is a zoonosis, and farm and companion-animals are significant reservoirs of the organism with the potential for transmission to humans. There is evidence that fresh meat, especially poultry, is a major source

of infection [164]. In addition, antibiotic resistant *C. jejuni* and *C. coli* are now being reported with increased frequency [86, 113]. Erythromycin and less commonly, ciprofloxacin, remain the agents of choice for the treatment of severe or recurrent *Campylobacter* enteritis in humans. However, it has been suggested by some investigators that fluoroquinolone and macrolide use in animals (for treatment and prevention) leads to the development of resistance among human isolates [38], whilst others suggested that resistance in *C. jejuni* and *C. coli* can be accounted for, at least in part, by use of antimicrobials to treat human infection. The association between the use of valuable drugs in veterinary medicine and the emergence of resistance in human isolates and *visa versa* requires a more complete understanding.

Treatment with antimicrobials is a risk factor for infection with organisms that are simultaneously resistant to several drugs and this may contribute to mortality [59]. Horizontal gene transfer is a significant mechanism for disseminating antimicrobial resistance among unrelated bacterial populations [10, 126]. Integron structures play a pivotal role and have been identified in several Gram-negative bacterial species including food-borne pathogens, such as *Salmonella* spp., *E. coli* and *Shigella* spp. [31, 138, 159]. Studies are now reporting the existence of these structures in *Campylobacter* and therefore their role and contribution to antimicrobial resistance must be assessed [76, 113].

Overall, amplifying the reservoir of resistance by whatever means is inherently problematic, making transmission to humans via food or other means more likely. Quantitative evaluation of associated risks will lead to the development and effective implementation of rational guidelines for antimicrobial use [59, 127]. Eliminating *Campylobacter* transmission via the food chain must remain a veterinary and public health priority.

8. *CAMPYLOBACTER* DETECTION

Campylobacter species and in particular *Campylobacter jejuni* and *Campylobacter coli* are the most common cause of gastroenteritis in humans in the developed world. Ever since its recognition as a cause of disease in humans, detection of this zoonosis has relied on culture-based methods. In fact, the original development of a culture media for the isolation of *Campylobacter* from human faeces by Martin Skirrow in 1977 [154] helped to firmly establish its role in human disease. Prior to this work, *Campylobacter* detection was reliant on membrane filtration of faecal samples onto non-selective media, a laborious and cumbersome method. Although Skirrow's medium was effective for isolating campylobacters from human faeces, it was less suitable for animal and environmental specimens, owing to the presence of contaminating species. This led to the development of the more selective Preston medium by Bolton and Robertson [19] suitable for the isolation of *Campylobacter* from foods and environmental samples. In subsequent years following these publications, further improvements have led to more sensitive and selective media for the improved detection of *Campylobacter* in faecal samples.

However, even with these improvements culture-based methods have a number of limitations. The methods are slow and in the case of human faecal samples require up to 48 h to yield a presumptive isolate, which then requires confirmation using phenotypic tests. In the case of food samples, where cell numbers can be low in a background of high numbers of other competing flora, enrichment culture in broth media is required to recover small numbers of cells prior to plating on selective media. This can lengthen the detection process with up to five days bring required to achieve a result. Culture-based methods may also select against less common species, such as *Campylobacter upsalienesis* and *Campylobacter lari,* leading to possible misdiagnosis and underestimation

of the true burden of infection with these other species.

The limitations of culture-based procedures led to the development of alternative methods for the detection of campylobacters in foods and faecal samples. The development of both poly and monoclonal antibodies specific for campylobacters has facilitated the development of a number of antibody-based tests. Latex agglutination tests for the identification of presumptive *Campylobacter* isolates have been developed, which can provide rapid more rapid species confirmation than conventional phenotypic tests [103]. A commercial enzyme-linked immunosorbent assay (ProSpecT Microplate assay; Alexon-Trend, USA) was developed for the detection of *C. jejuni* and *C. coli* directly in faecal samples, from humans with gastroenteritis [174]. This assay was demonstrated to have a sensitivity of 96% and a specificity of 99% when applied to 50 *Campylobacter* culture-positive and 114 culture-negative faecal specimens [174]. A second prospective study of 1205 faecal samples demonstrated similar results with a sensitivity of detection of 97.7% [32]. The assay also provided more rapid results when compared to conventional culture, with results being available within hours rather than days. Such rapid methods may prove useful in cases where early diagnosis may alter patient management or treatment.

Since its discovery PCR has impacted on virtually all areas of microbiology and in particular has been used to detect microbial pathogens in a wide range of sample types. The first application of PCR for the specific detection of *C. jejuni* and *C. coli* was described in 1992 [123]. The assay targeted the flagellin A gene of *C. jejuni* and *C. coli* and was demonstrated to be specific for these two species and successfully detected 30–60 bacteria per PCR reaction in seeded human faecal samples. This report also demonstrated the potential of the PCR-based methods to detect very low numbers of *Campylobacter* cells. However, complex sample preparation methods and the use of gel electrophoresis end-point detection methods, requiring manipulation of amplification products following PCR cycling, hampered the transition of these methods from research to routine microbiology laboratories. Adaptation of PCR assays into a microplate hybridisation format or PCR-ELISA increased the specificity and sensitivity of detection. Lawson et al. [71] developed a panel of PCR-ELISA assays which they used in a large-scale survey of the detection of *Campylobacter* species in human gastroenteritis. The assays could detect and differentiate between *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. helveticus*, *C. fetus*, *C. hyointestinalis* and *C. lari* with the PCR-ELISA results being compared with conventional culture methods. The PCR-ELISA assays detected campylobacters in culture-negative faecal samples and also more importantly, detected mixed infections with more than one *Campylobacter* species. The assays did provide information on the identity and occurrence of species that are not detected by culture, however the authors did note that PCR was more expensive and labour-intensive than culture. The use of such assays may prove useful in further large-scale epidemiological surveys of *Campylobacter* infection and provide evidence of the role of *Campylobacter* species, other than *C. jejuni* and *C. coli,* in human disease.

The first report of a PCR assay for the detection of campylobacters in foods was made by Giesendorf et al. [54] who described a PCR assay for the rapid and sensitive detection of *Campylobacter* species in chicken products. The assay was applied to the detection of *Campylobacter* species in both naturally contaminated and artificially inoculated samples of chicken skin following enrichment culture of the samples for 18 h in Preston enrichment broth. The assay demonstrated a limit of detection of 25 CFU of *Campylobacter* species per gram of tissue following the 18 h enrichment. The use of PCR for the detection of *Campylobacter* in foods is hampered by the relatively large sample size (25 g in most

routine test procedures) compared to the final template volume in the PCR assay (often 1–5 μL). For a PCR assay to replace conventional culture methods, it must have a limit of detection sufficiently sensitive to be able to detect a single *Campylobacter* cell in 25 g food. In order to biologically amplify the numbers of cells present, many PCR-based studies have utilised enrichment culture prior to application of the PCR assay. There have been many further reports of PCR assays for the detection of campylobacters in a range of sample types including foods, environmental waters and other environmental samples. Although these assays may be useful as an adjunct to enrichment culture, by reducing the total time of detection by two or more days, they are still limited by inefficient sample preparation methods. Many PCR assays have a limit of detection of a single cell per reaction, however the inability to separate low numbers of cells away from the sample matrix remains the "bottleneck" for the adoption of PCR-based methodologies in routine food microbiology testing laboratories. Specific and sensitive methods are required to separate the target cells away from the sample matrix in a form amenable for PCR-based detection. In a recent study, paramagnetic beads were utilised as a method for isolating *Campylobacter* from chicken cecal contents and faecal samples, prior to PCR [139]. The beads initially bound to the cells in the liquid sample matrix and then lysis buffer was added to lyse the cells releasing the DNA, which then also bound to the beads. The DNA was then washed and used as template in the PCR assay. This procedure may prove useful in food testing laboratories, however further studies are required to validate this approach for food sample testing.

The introduction of real-time PCR methods have facilitated the development of quantitative PCR assays for the detection of *Campylobacter* in foods [145], milk and environmental waters [194]. The assays demonstrated a range of quantitation over 6 orders of magnitude with the results correlating closely with culture. The quantitative detection of *Campylobacter* directly in raw-meat rinse fluid samples was also demonstrated however the limit of detection was compromised by the presence of PCR inhibitors and the low numbers of cells present (Sails et al., unpublished data). The use of sensitive, quantitative PCR methods for the detection of *Campylobacter* during food processing could be used to determine points in the food production process where contamination occurs and where controls could be introduced to reduce or eliminate *Campylobacter* from retail food products, thereby reducing the risk to the consumer.

The ultimate goal of nucleic acid based detection methods is to facilitate direct detection of pathogens in food samples without the need for enrichment culture. This would permit more rapid detection of pathogens, thereby reducing the time of detection to hours, rather than days. In order to determine the viability of the detected pathogen, the assay must target a cellular process or molecule, which has been shown to be associated with bacterial viability under all conditions tested. Conventional PCR methods detect chromosomal gene sequences, which can be present in non-viable cells. Therefore, direct detection by conventional PCR cannot determine the viability of the detected cells somewhat limiting the usefulness of such methods in food microbiology testing. Detection of viable cells using messenger RNA (mRNA) as the target for reverse transcriptase PCR (RT-PCR) has been investigated for several microbial pathogens including *C. jejuni* [144]. The RT-PCR assay was demonstrated to differentiate between viable and heat-killed cells of *C. jejuni,* however the method of killing and post-treatment holding conditions did influence the rate of mRNA degradation in the cells. Further studies are required to investigate the effect of different killing methods and post–treatment holding conditions to determine the factors which influence the rate of mRNA degradation in dead cells. This will allow the above factors to be

investigated and the results related to food processing methods.

Campylobacter detection methods have improved significantly since the initial isolation of campylobacters using membrane filtration and non-selective media in the early 1970s. Improvements in molecular methods have facilitated the development of nucleic acid-based detection methods which are more rapid, sensitive and specific. In the future, improvements in sample extraction methods allowing more sensitive detection of cells by PCR will facilitate the uptake of these methods by microbiology laboratories. Eventually, biological growth or amplification in vitro may be replaced with DNA amplification, with culture media being replaced by PCR reagents and the incubator being replaced by the thermal cycler.

9. CONCLUSIONS

The past three decades have witnessed the rise of *Campylobacter* enteritis in man from virtual obscurity to notoriety, with present isolation rates superseding those of other enteric pathogens such as *Salmonella* spp. and *Shigella* spp. in most developed countries. Unlike the salmonellae and other enteric pathogens, the majority (ca. 99%) of clinical reports concerning *Campylobacter* are sporadic and *Campylobacter* enteritis outbreaks are rare. The lack of well-developed typing schemes has hindered the epidemiological investigations seeking natural reservoirs of the organism and modes of transmission from these sources to man. Only about 15% of clinical isolates are identified to species level thus making epidemiological investigations extremely difficult to perform. Since the development of more sophisticated isolation techniques, the true disease potential of these organisms has become apparent and today campylobacteriosis is regarded as a zoonosis, which is capable of being transmitted to man by a wide range of domestic animals. Presently, the laboratory isolation of these organisms has become routine from both clinical as well as from environmental specimens and although relatively complicated to perform, routine isolation has been carried out with success for this past 20 years or so.

Campylobacter spp. are the most common cause of acute gastroenteritis in the developed world. Thermophilic campylobacters, i.e. those *Campylobacter* spp. which are able to proliferate at 42 °C, particularly *C. jejuni, C. coli* and *C. lari*, are of particular interest to the food industry, as these campylobacters form the natural microflora of the gastrointestinal tract of several domestic and pet animals including poultry. Although campylobacters are the most common cause of acute human enteritis, their routes of infection and transmission to man are still not fully understood. Further work is still required to find the source(s) of these organisms of major public health concern.

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