ABSTRACT

During the 2002-2006 periods two bilateral government scientific projects between R Slovenia and R/F B&H related to *Campylobacter* epidemiology and antimicrobial resistance took place, resulting in significant results. In this review key findings relating to *Campylobacter* epidemiology in Zenica-Doboj Canton, Bosnia and Herzegovina, were presented.

**Key words:** *Campylobacter jejuni*, *Campylobacter coli*, fluoroquinolone, erythromycin, PFGE, efflux pump inhibitors
INTRODUCTION

Campylobacter jejuni and Campylobacter coli are still some of the most important enteropathogens worldwide (1-3). Understanding of their epidemiology is complicated by the sporadic nature of the disease, a lack of representative population sampling (4), wide distribution in the environment (5), and a high level of genetic diversity (6,7). The major route of infection in humans is through consumption of contaminated poultry meat, probably because of contamination of chicken carcasses with Campylobacter and the frequency of poultry consumption (3,8,9). Transmission to humans also occurs via other types of food, drinking water, and pets (8,10).

The first observation of Campylobacter-like microorganisms as agents of a disease dates more than a century ago. In 1886, Escherich reported the uncultivable Campylobacter-like forms in stool samples of children with diarrhea (11). C. jejuni (Vibrio fetus subsp. jejuni until the reclassification in 1963) was first isolated in 1909, and it was associated with abortions in sheep and cattle. In 1947 it was cultured from human blood (12). Over the next decade it was recognized as an opportunistic pathogen of immucompromised patients (13,14). The possibility that V. fetus could be also associated with enteric disease was first postulated by Elizabeth King, who in 1957 divided it into two groups based on thermophilic characteristics. The bloodstream isolates that grew best at 42 °C were originated from patients with preceding diarrheal illness (15, 16). Since then, many epidemiological studies, particularly those of Butzler and Skirrow (17), Blaser et al. (18), and Skirrow (19) have confirmed the importance of King’s ‘related vibrios” as one of the major bacterial enteric pathogens worldwide.

EPIDEMIOLOGY

The first investigations in former Yugoslavia

The first investigations in former Yugoslavia about an extent of Campylobacter among cattle originated from 1956 Frans et al. work from Zagreb (Croatia) (20), and after that from Brgles et al. from Ljubljana (Slovenia) in 1962 (21). Many works relating to this issue were done by Mehle (22). Except the best knowledge about Campylobacter in veterinary medicine originated from Slovenian researches, there were, also from Slovenia, early works about human campylobacteriosis done by Blatnik-Gubina in 1971 (23) and 1977 (24,25). Janc and Satler-Zaje in 1981 completed the role of Campylobacter in enteric human disease (26).

In Croatia, the first investigation of the role of Campylobacters in human enteric disease originated from Kalenić et al. in 1982 (27). Popović-Uroć give more complete picture of human campylobacteriosis at the Zagreb area in 1986 (28).

The first investigations in Bosnia and Herzegovina

In the Bosnia and Herzegovina Uzunović-Kamberović published in 2001. the first prospective study assessing carriage rate of Campylobacter jejuni and Campylobacter coli in the Zenica region (Bosnia and Herzegovina), in the view of the socio-economic changes resulting from war-associated events. The study covered two distinct time periods, the pre-war (1990-1991) and the post-war (1996-1998) period, giving a carriage rate of 0.8% and 0.1% respectively (29).

Fortunately, in the period 2002-2008 we got two bilateral governmental (FB&H and Slovenia) projects with Slovene research group, so in these years we have been working on (molecular) epidemiology and antimicrobial resistance (mechanisms) in campylobacter from different sources in both countries (30-38).

INCIDENCE OF CAMPYLOBACTER INFECTIONS

Reportedly, the incidence of Campylobacter infections in northern European countries has been 60-90/100.000 inhabitants. Since confirmed and reported infections represent only a fraction of the total number of the actual infections, the true incidence could be 10-100 fold higher (39). In twelve member states of European Union, in overall, there has been an increase by 33% of the reported cases of human campylobacteriosis during the years 1996 and 2000 (40). In The United States and in a number of developed European countries (Scandinavia, the Netherlands, Ireland, United Kingdom) the reported incidence rates for campylobacters surpassed those of salmonella from patients suffering gastroenteritis (40-42). The exact reasons for the increasing incidence of human campylobacteriosis in developed countries are not known. Better surveillance systems
and diagnostics could have one of the reasons (42). The changes in consumption of some foods, particularly fresh poultry meat could be other important reason of Campylobacter incidence increase (43).

In the 1999-2001 period surprisingly low number of isolates, and accordingly, significantly decrease of campylobacter infection incidences in the Zenica-Doboj Canton were recorded, 7.5, 1.8, and 6.6 per 100,000/year, respectively (29), compared with the years 1998 and 2002, 18.3 and 16.0 per 100,000/year, respectively (31) given that the data source were from the same laboratory (the same method and staff). The similar situation was reported for a decrease in campylobacter carriage rate after the war comparing the pre-war period in this region (29). Decreasing number of Campylobacter infections was also observed in some other reports (1, 28). Different reasons were attributed to disease prevention efforts (1), or changes in nutritional habit of the population (28). In the same period of time, the number of Salmonella infections increased, so than the overall incidences of these two microorganisms were relatively constant during 1998-2000 period (31,44). It might be some kind of natural counterbalance of these pathogens (1).

As it was stated before, some year-to-year variation in incidence of food borne disease can be attributed to the prevalence of pathogens in their respective important animal reservoirs and the foods derived from them (1,44). The reason for the variation in incidence of the two pathogens in the humans might be different epidemiology, origin and transmission cycle of the two microorganisms. All Campylobacter infections were sporadic in our region, but 20% of Salmonella infections appeared in small-family outbreaks (44). While an epidemiology, natural reservoir and transmission of the most important Salmonella spp. are well established (45,46), for Campylobacter spp. they are still poorly understood (47). It is because of the complicated epidemiology of this zoonosis, by sporadic nature of the disease, organism’s wide distribution, high level of genetic and antigenic diversity, and lack of representative population samples (7).

Although campylobacter isolation rate was low in this region, as in industrialized countries, both, the highest incidence and isolation rate of infections in children fewer than 6 years of age (unimodal age-specific distribution) are epidemiological features similar to that of developing countries (31,48). However, irregularities of sampling have influenced the isolation rate. This bias is inherent in all laboratory-based surveillance studies, and reflects actual clinical practice. Most surveys in developed countries showed bimodal age-distribution of infection (49). This unimodal age-specific distribution might be also consequence of recent war in this region (31). Crowded urban community and poor sanitary conditions influence spreading of infections to be highest in young children (29,50). Although the youngest are the most affected population in Salmonella infections in this region as well, there was no strictly unimodal age distribution persisted, but incidence of Salmonella infections rather have had decreasing trend according to age (44).

**TRANSMISSION OF CAMPYLOBACTER INFECTIONS**

Different vehicles for transmission of human Campylobacter infections have been identified, but it is estimated that around 80% of them are transmitted by food (41,51). Undercooked or cross-contaminated poultry meat, untreated drinking water, and raw milk have been sources of Campylobacter outbreaks (43,52). However, the vast majority of Campylobacter infections are reported as sporadic individual infections with no identified source of infection (53-55).

It was proposed that the single most important route of Campylobacter infections (50%-70%) in industrialized countries remains consumption and handling of chicken (33,56,57). This should not be surprising in the light of the frequency with which poultry products are consumed and nearly universal contamination of chicken with Campylobacter spp. (31,56,57).

Unfortunately, epidemiological data in 2005 study from this region obtained from only 34% of patients, 22.2% of which connected their illness with particular food items (poultry and beef meat), and 50% have had a contact with an animals (pets, chicken and cows). Therefore, strong epidemiological link between the cases and potential sources of infection missed (31).

It was reported from several European countries that the prevalence of Campylobacter spp. on raw chicken meat from retail sail could vary in
relation to the geographic location, season and method of isolation, etc., but was frequently as high as 80% (43, 58-62). The extent of poultry meat contamination with Campylobacter spp. in Slovenia and Bosnia and Herzegovina was 73% and 45.5%, respectively, and 65.2% in Bosnian farm animals (farm chickens) (9,33,63).

*C. jejuni/*C. coli*

*C. jejuni* is responsible for more than 90% of the cases. Although *C. coli* accounts for a minority of human Campylobacter infections, its health burden is greater than previously thought (3,64). However, in Croatia and Bosnia and Herzegovina, the clinical implication of closely related thermophilic *C. coli* was much higher, 30-40% (4,28,29,65). The majority of *C. coli* isolates analyzed in large number of studies was obtained from pigs, suggesting that pigs were the most probable source of human infections with *C. coli*, rather than chicken and cattle (66). Early works from Zenica-Doboj Canton (4,29), showed a higher isolation rate of *C. coli* in clinical material than in other countries (7,28,66) suggesting that the primary source of *C. coli* infections might be other than pigs. Such distribution of *C. jejuni/*coli* infections were shown only in a few reports. In Croatia excess of *C. coli* infections (54%) was probably the consequence of the local tradition of home slaughter and processing of pigs at the end of the summer, and the fact that *C. coli* is particularly associated with pigs (28). In the Central African Republic (39%) an individual well-water supply was identified as a possible source of *C. coli* infection (67). It was reported also from England and Wales sentinel surveillance scheme that risk factors for infection by *C. coli* are different from those for infection by *C. jejuni* (68).

**MOLECULAR GENOTYPING METHODS**

For studying the epidemiology of Campylobacter infections, several genetic typing methods have been developed in order to differentiate isolates below species level (7,9,69,70). Pulsed field gel electrophoresis (PFGE) typing analysis is a highly reproducible and discriminatory technique allowing a comparison between PFGE patterns in human isolates and isolates obtained from other sources (7,10,69,71). Identical genotypes found in poultry products and humans might indicate common sources of infections and provide data on the genotype stability (64,72). Although most reports based on molecular typing have shown the role of poultry consumption in human Campylobacter infection, its epidemiology is still not completely defined (70).

The results of the study conducted during 2001-2002 in Zenica-Doboj Canton demonstrated a significant proportion of *C. coli* among human, poultry meat and farm chicken isolates. Besides, large heterogeneity of genotypes among *C. coli* and *C. jejuni* isolates were identified. Identical PFGE genotypes were found in small proportion of humans and poultry meat isolates, as well as in poultry meat and farm chicken isolates (33). PFGE showed that poultry meat may be the source of sporadic Campylobacter infections (2,32,69).

However, PFGE- and RFLP-typing analysis of human and poultry meat isolates in this region, where only a minority of clinical and poultry meat isolates of *Campylobacter* shared identical PFGE types, showed that the sources other than poultry might be important. All sub clusters were unified according to the species, but not according to the isolation source or geographical affiliation, and therefore, the potential sources of the majority of clinical Campylobacter infections were difficult to identify. High prevalence of *C. coli* isolates from humans, poultry meat, and farm chickens suggests, however, that there may be a common source in the environment, which might be absent in other geographical regions. We have not yet been able to explain the high prevalence of *C. coli* human infections in this region (33).

Some authors suggest that *C. coli* chicken and porcine isolates represent host-specific populations (8,73,74), while others suggest that some *C. coli* strains have a wider host range than others (75). One study has shown that the majority of *C. coli* isolates from pigs were distinct from human isolates, and accordingly, that pork might be an infrequent source of human Campylobacter infections (75). Pigs, however, remain a potential source of *C. coli* infection for persons who come into close contact with them (8). On the other hand, since some chicken *C. coli* isolates have shown identical fingerprints with human *C. coli* isolates, it has been proposed that not only chickens but other poultry sources such as turkeys, ostriches, and ducks may be a transmission vehicle of *C. coli* to humans (75).
The question arises whether some clones are so common in the environment that they can infect farm chickens in different geographic areas (Bosnia and Herzegovina, Slovenia) by some, as yet unexplained, transmission route, such as through wild birds or by sharing a previously infected source of breeders for broiler production in these countries (76).

ANTIBiotic RESISTANCE

As Campylobacter may be transferred from animals to humans, the possible development of antimicrobial resistance in Campylobacter spp., due to the use of antimicrobial agents in food animals, is a matter of concern (77,78). Studies from northern Europe have associated fluoroquinolone use in food animals, particularly in poultry, as the source for fluoroquinolone-resistant Campylobacter spp. human infections (78).

It is generally accepted that the main risk factor for accumulation of resistant bacteria is an extensive use of antibiotics, not only for therapy and prevention of infections in humans and animals, but also for usage of sub-therapeutic doses in animal feed to promote growth, increase feed efficiency and decrease waste production (30,34, 79-82). Despite legislation targeted at controlling the use of antimicrobials in food-producing animals in recent years there has been significant increase in developed countries in the occurrence of resistance to antibiotics in different food-borne bacteria including Campylobacter spp. (83). Zoonotic bacteria can transmit antibiotic resistance genes from the food-producing environment via food directly to the consumer (84).

A useful tool to confirming the link among emerging fluoroquinolone resistance of Campylobacter from food animals, foods of animal origin and human isolates are microbial typing methods, such as automated ribotyping (RiboPrinting), pulsed-field gel electrophoresis (PFGE) using different restriction enzymes and some PCR-based typing methods (85). They give stable profiles of isolates from different sources over time (86). Some authors confirmed identical or very similar fingerprints of resistant campylobacter’s from animal and human sources (87-89), but more research is needed to elucidate as well the importance of other reservoirs of resistant strains, possibly common to food animals and humans, like wild animals, pets, waters (90-93).

Macrolide resistance of Campylobacter spp. has already been studied in many countries but only in few reports an increase was documented for humans, mainly C. jejuni isolates (94,95).

It is an usual feature of C. coli strains to be more resistant than C. jejuni (57,90). High overall erythromycin-resistance rate of Campylobacter spp. isolates (32%) persisted in this region since 1998 (25%) (30). In most other reports erythromycin resistance is up to 2% (28,95). C. coli strains have usually shown ten times higher resistance rate to erythromycin than C. jejuni (28,57). This difference was much lower in this region: 46.3% of C. coli isolates were erythromycin-resistant and 26.3% of C. jejuni. In the 1998 survey the erythromycin-resistance in the two species was almost equal (30). Such extremely high erythromycin-resistance rates could be comparable only with the report from Spain (57).

The highest rates of fluoroquinolone-resistant Campylobacter spp. have been reported from southern Europe, and other regions (28,30,57,78, 95). In this region, the frequency of resistance to ciprofloxacin in the youngest for which fluoroquinolone use is not recommended, was surpassingly high (4,31,34). Thus, overuse of this drug probably was not the reason for such a high frequency of resistance. Some reports indicated that the source of fluoroquinolone-resistant Campylobacter infections was the consumption of poultry colonized with resistant strains, rather than selection for Campylobacter resistance in the human gut after clinical fluoroquinolone use to threat diarrheas (95).

The increasing resistance to fluoroquinolones was reported also for clinical isolates of Campylobacter spp. in Slovenia and Bosnia and Herzegovina (30,34,82). We compared the results of antimicrobial susceptibility testing of campylobacter from retail poultry meat and human clinical isolates collected in the same time frame (2001-2003). Antimicrobial susceptibility testing against eight different antibiotics used in veterinary and/or human medicine was included in the study. The portion of ciprofloxacin resistant human clinical isolates has increased in Slovenia as well as in Bosnia and Herzegovina from 10.5% and 15% in the year of 1998, to nearly 50% and 32% in 2002, respectively. However, while ciprofloxacin resistance of clinical isolates in Slovenia
still lower than in poultry meat isolates (58.2% of resistant strains among poultry isolates from years 2001-2003), in Bosnia and Herzegovina the situation was different: Campylobacter isolates from poultry meat and farm animals had lower extent of ciprofloxacin resistance (27% and 31%, respectively) than human isolates (34,98).

The resistance to ciprofloxacin, beside E-test, was also confirmed by MAMA-PCR detecting mutations in quinolone resistance determining region (QRDR) of the gyrA gene of C. jejuni and C. coli (99). These results indicate that poultry meat was indeed an important source of antibiotic resistance in thermotolerant campylobacter. Moreover, multiple antibiotic resistance was found as a critical point of tested food isolates (100). Our results showed that we need a monitoring system of the prevalence and antibiotic resistance of zoonotic bacteria from human, animal and food samples on a national or regional level to better understand the epidemiology of animal, food and human strains and to assure safety of food products.

The pig is the favored host of C. coli and historically has shown to have a high resistance rate to macrolides. Macrolide tylosin has been permitted as a growth promoter in pigs, but not in broilers, and this could explain the lower proportion of erythromycin-resistant strains observed in broilers than in the pigs (57). Given that C. coli strains in previous investigations from our region probably did not originate from pigs, our observation of high prevalence of erythromycin-resistance is unexplained. The differences in isolation rate of the different Campylobacter species among different sources may make it difficult to compare levels of resistance between the sources. Thus, it is at present difficult to say whether the higher level of macrolide resistance among C. coli isolates is because of their origin (most isolates are from pigs) or it is related to true differences among the species (90).

Erythromycin and fluoroquinolones are considered to be the drugs of choice for the treatment of Campylobacter infections. An increase of antibiotic resistance of human Campylobacter isolates, especially to fluoroquinolones has been reported in many countries, but resistance to erythromycin and other antimicrobials has also been observed (30,96,101). As Campylobacter may be transferred from animals to humans, the possible development of antimicrobial resistance in Campylobacter spp., due to the use of antimicrobial agents in food animals, is a matter of concern (101,102).

Mechanisms of antibiotic resistance

Finally, we investigated Campylobacter jejuni and C. coli from poultry, water and human clinical samples collected during the period 2001-2007 in different geographical areas (Slovenia, Bosnia and Herzegovina) and characterized by classical and molecular identification and typing methods (mPCR, fla-RFLP, PFGE). The differences in macrolide resistance (against erythromycin, azithromycin, clarithromycin) were studied by disk diffusion, E-test and broth micro-dilution method. The mechanisms of macrolide resistance and its stability was studied phenotypically by broth micro-dilution test with and without different efflux pump inhibitors and by molecular methods like PCR-RFLP of target genes and their sequencing analysis (35,36,38).

Additionally, we compared the occurrence of antimicrobial resistance to ciprofloxacin, erythromycin and tetracycline among poultry meat and water isolates, identified as C. coli, C. jejuni or Campylobacter spp. Resistance to ciprofloxacin was more frequent among meat isolates (43.8% versus 26.0% among water isolates). In contrast, resistance to erythromycin was much more frequent among water isolates (44.0% versus 21.4% among meat isolates). However, the erythromycin resistance rates of meat and water isolates were very high, comparing to reports from some other European countries. Tetracycline resistance was rare among water isolates, but quite frequent among meat isolates (6.0% versus 18.8%, respectively).

According to the mechanisms of resistance involved, the resistance of isolates to erythromycin, ciprofloxacin and tetracycline was studied in the absence and presence of efflux pumps inhibitor phenylalanine-arginine β-naphthylamide (PAβN), which affected the efflux pump CmeABC. As the result of PAβN presence, the susceptibility to erythromycin was in average increased 24-fold. The presence of efflux pumps activity at the isolates with mutations in target genes was also observed and proved synergistic activity of these two drug resistance mechanisms. The smaller effect of the PAβN was also observed in the use of ciprofloxacin and tetracycline (35,36).
Unusually high prevalence of C. coli and the resistance to macrolides in both C. jejuni and C. coli isolated from humans, poultry, farm chickens, and water was noted. Efflux activity was confirmed as involved mechanism at erythromycin, azithromycin, clarithromycin and clarithromycin resistance. However, the results confirm that PAB’N and NMP efflux pump inhibitors were moderately active in reversing macrolide resistance in Campylobacter isolates. Further studies of mechanisms involved in macrolide resistance of Campylobacter is required, as well as monitoring of macrolide resistance of human, animal and environmental isolates (37).

The resistance of Campylobacter strains to other macrolide antibiotics (azithromycin, clarithromycin, tylosin), the efficiency of other chemical efflux pumps inhibitors (NMP, some compounds from natural plant extracts) as well as monitoring of macrolide resistance of human, animal and environmental isolates are needed to improve the understanding of mechanisms involved in Campylobacter resistance and provide new options for improved safety in food production and supply (103).

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TRANSPARENCY DECLARATIONS

Competing interests: none to declare.

REFERENCES


SAŽETAK

U periodu od 2002. do 2006. godine, u sklopu dva znanstvena bilateralna vladina projekta između Republike Slovenije i R/F BiH, sprovedena su istraživanja slovenske i bosanske grupe istraživača koja su rezultirala značajnim rasvjetljavanjem epidemiologije i antibakterijske rezistencije Campylobacter spp. u Zeničko-dobojskom kantonu. U ovome preglednom članku prikazani su najvažniji rezultati dobijeni tokom istraživanja.

Ključne riječi: Campylobacter jejuni, Campylobacter coli, fluorokuinoloni, erithromicin, PFGE, inhibitori efokusne pumpe