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Original article

Prevalence and antimicrobial resistance of *Campylobacter* in raw milk in the selected areas of Poland

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Abstract

During the recent years, an immense increase in the number of food poisoning cases in people caused by *Campylobacter* (*C.*) species has occurred. Raw milk, next to poultry meat, is considered the most frequent cause of food poisoning in people caused by the subject bacteria, although it is not always possible to isolate *Campylobacter* cells from the incriminated milk. Most probably this difficulty is caused by low concentration of the pathogen in milk at the level of 2/3 cells/ml although even such low concentration represents risk to human health. The present study was aimed at determining the occurrence of *Campylobacter* bacteria in milk originating from selected regions of Poland. The isolation method applied in this work was effective in recovering as few as 0.1 cell of *Campylobacter* per g of food. Among 150 bulk milk samples tested, *Campylobacter* spp. was isolated from 7 (4.6%) ones. The biochemical identification of the isolated strains conducted by means of conventional biochemical tests as well as by applying the API – Campy tests revealed that all the isolates belonged to the *C. jejuni* species. Determination of resistance to antibiotics was performed by means of the diffusion disks method for the following antibiotics: gentamicin, ciprofloxacin, ampicillin, chloramphenicol, erythromycin, doxycyclin and tetracycline. Among 7 isolates tested, all were susceptible to ampicillin, chloramphenicol, erythromycin and gentamicin, 28.5% to doxycyclin and 14.2% to tetracycline and ciprofloxacin.

Key words: *Campylobacter*, contamination, antibiotic resistance, raw milk

Introduction

Health of dairy cattle herds and milking conditions are the basic factors conditioning microbiological milk quality. In most cases, if the animal is not suffering from an intramammary infection or a systemic disease, milk inside the mammary gland contains no bacteria, although during milking the milk

can be contaminated with microorganisms living on the skin of the teats (White et al. 1989). Additionally, the farm environment is an equally important source of many microorganisms responsible for foodborne diseases. The presence of foodborne pathogens in milk represents a potential threat to public health, particularly among consumers of raw milk – milk producers, farm workers and their families or people

keen on drinking raw milk (Ryser 1998). It is estimated that during the years 2007-2008, 21-22% of the milk produced was used at farms while 78% of the global production was sold of which 5-7% was sold through direct sales. It can be assumed that the milk consumed at the farm as well as the milk for direct sale was consumed mainly as raw milk (Seremak-Bulge 2008). In Poland, similar to the other EU countries, sale of raw milk is legal. Additionally, the regulations concerning hygiene applicable to the food of animal origin allow for sale only the milk containing no more than 100,000 microorganisms per 1 ml originating from farms that are under veterinary control and satisfy the structural sanitary requirements. The above requirements, however, do not guarantee that the raw milk is pathogens free. Raw milk is an ideal medium for growth of microorganisms, including pathogens responsible for foodborne diseases including *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Bacillus cereus* and *Campylobacter* spp. Gastritis and enteritis are the major foodborne diseases resulting from consumption of raw milk (Jayarao et al. 2006).

Genus *Campylobacter* bacteria are one of the main etiological factors of gastroenteric diseases in people manifesting as foodborne infections in most cases involving the symptoms of diarrhea. The widespread presence of this pathogen in animal population involves the risk of animal products contamination. Raw or insufficiently cooked poultry meat is considered the most frequent cause of *Campylobacter* related cases of food infections in people. Next to the poultry, raw milk is considered an important vector of *Campylobacter* spp. infections in people (Skirrow 1982, Stadler et al. 1983). Raw milk was described frequently as the vector of foodborne enteritis caused by *Campylobacter* spp. (Finch and Blake 1985, Hargrett-Bean et al. 1988), although it was not always possible to recover this microorganism from the contaminated milk. Most probably this difficulty is caused by a low concentration of *Campylobacter* cells in raw milk. On the other hand, even low concentrations of this pathogen represent hazard to human health (Christopher et al. 1982). Studies conducted by Robinson (1981) showed that just 2-3 *C. jejuni* cells/ml of milk may contribute to infection in humans and cause symptoms of gastroenteritis, which supports the need for a procedure for rapid detection of low numbers of *Campylobacter* in food. The isolation method applied in this work was effective in recovering as few as 0.1 cell of *Campylobacter* per g of food.

Additionally, the tests conducted aimed at determining the susceptibility of genus *Campylobacter* bacteria to antibiotics. During the recent years, an increase in resistance to known and generally applied therapeutic

drugs has been observed, which seems to pose a serious problem appearing in treatment of bacterial foodborne diseases.

Materials and Methods

Isolation and identification

The test material consisted of 150 samples of cowshed collected raw milk originating from farms situated in the Warmia and Mazury as well as Mazowsze regions (Poland). The samples were stored at 4°C and tested within 6 h after sampling. The isolation and identification of thermotolerant *Campylobacter* species was conducted according to the method described by Doyle and Roman (1981) and recommended by the Food and Drug Administration (Hunt et al. 2001). 25 ml of each raw milk sample was added to test tubes containing 225 ml Bolton bullion as liquid culture medium. The suspension obtained was incubated in microaerophile atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C for 4 h, and next at 42°C for 44 ± 4 h. The culture obtained on the culture medium was transferred using sterile loop to the surface of two parallel selective agar media: mCCDA (modified *Campylobacter* Blood-Free Selective Agar Base, Oxoid) and Karmali (Oxoid). The plates were incubated at 41.5°C in microaerophile atmosphere. After 44 h ± 4 h incubation test plates were checked for the presence of colonies suspected of belonging to genus *Campylobacter*. Characteristic grayish, flat, moist colonies with the tendency for overflowing growth were analyzed under contrast-phase microscope (1500 x magnification). Spiral cells showing plane-rotary motion were confirmed as being *Campylobacter* cells. The isolated strains were subject to species identification by means of two methods: conventional biochemical tests according to the PN-ISO 10272-1 and API – Campy tests (BioMerieux) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

All the pathogens isolated confirmed as belonging to *Campylobacter jejuni* species were subjected to tests for determination of susceptibility to antibiotics. The tests were conducted according to the diffusion – disk method following recommendations by the National Committee for Clinical Laboratory Standards (NCCLS). To obtain pure culture, each selected colony was suspended in Brucella broth with the density of 0,5 according to McFarland scale and next diluted 1:10. The received suspension was poured on the sur-

face of Mueller-Hinton medium supplemented with 5% of blood. Next disks soaked in the antibiotic were placed on the surface of the medium. The following antibiotics were tested at the specified concentrations: erythromycin (15 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), doxycycline (30 µg). Plates were incubated at 37°C for 22 ± 2 h at microaerophile atmosphere. Zones of inhibited growth were determined according to the NCCLS standards.

Results

Among 150 raw milk samples tested the presence of thermotolerant bacteria of genus *Campylobacter* were found in 7 representing 4.6%. Identification of the isolated *Campylobacter* spp. strains by means of both the conventional biochemical tests and on the base of API – Campy showed that all the strains belonged to *C. jejuni* species. On the base of identification of the isolated strains by means of conventional biochemical tests, the oxidase and catalase positive strains, susceptible to nalidixic acid and resistant to cephalotin, capable of hydrolyzing hippurane and indoxyl acetate were confirmed as *C. jejuni*. Confirmation of isolates using API Campy test covered the characteristic reaction of hippurane and indoxyl hydrolysis as well as urease activity.

Among the identified *Campylobacter jejuni* strains no strains resistant to ampicillin, chloramphenicol, erythromycin and gentamicin were recorded while 28.5% of strains were susceptible to doxycycline and 14.2% to ciprofloxacin and tetracycline. Five (71.4%) isolates were resistant to three or more antibiotics while one isolate was resistant to one and two antibiotics. The results of tests determining the susceptibility of isolated *Campylobacter jejuni* strains to antibiotics based on the diffusion-disk method are presented in Table 1.

Table 1. Antimicrobial susceptibility of 7 *Campylobacter jejuni* isolates from milk of dairy cows.

Antibiotic	n/N	%
Ampicillin	7/7	100
Ciprofloxacin	1/7	14.2
Chloramphenicol	7/7	100
Doxycycline	2/7	28.5
Erythromycin	7/7	100
Gentamicin	7/7	100
Tetracycline	1/7	14.2

N – number of isolates tested

n – number of isolates susceptible to a given antibiotic

Discussion

The isolation ratios similar to those obtained during own studies were also obtained by Larkin et al. (1991) and Beumer et al. (1988) recovering *Campylobacter* sp. from 2 (5.0%) out of 41 raw milk samples as well as 41 (4.5%) out of 904 raw milk samples respectively. Lower levels of milk contamination were recorded by Doyle and Roman (1981), who confirmed the presence of *Campylobacter* bacteria in 1 out of 108 raw milk samples and Gomółka and Uradziński (1996), who found *C. jejuni* in 2 out of 130 raw milk samples (1.5%). Similar results were obtained by Manus and Lanier (1987), who analyzed 237 raw milk samples and recovered *Campylobacter* in only 1 (0.4%). A higher recovery rate at the level of 12% and 12.3% were obtained by Humphrey and Hart (1988), Rohrbach et al. (1992) as well as Jacobs – Reitsma (2000).

In the present study research the identification of *Campylobacter* strains showed that all the strains belonged to *C. jejuni* species. Also Yaman and Elmali (2004) isolated *Campylobacter* from 6 (5.0%) out of 120 samples tested and all the strains were confirmed as *C. jejuni*. In the studies conducted by Hussain et al. (2007), genus *Campylobacter* bacteria were observed in 10.2% of the raw bulk milk samples and 92.4% of the isolates were identified as *C. jejuni* while 7.6% as *C. coli*. According to the available literature, among *Campylobacter* species, *C. jejuni* and *C. coli* are commonly found worldwide, of which *C. jejuni* is responsible for 80 – 90% of human infections while *C. coli* for ca. 7%, and *C. lari*, *C. hyointestinalis* and *C. upsaliensis* for the remaining cases (Nesbakken et al. 2002).

Milk contamination during or immediately after milking is, most probably, of stool origin. The alimentary system of cattle is a significant reservoir of *Campylobacter* spp. (Prescott and Bruin-Mosch 1981, Stern 1981), while the level of carrier state in herds of dairy cows is estimated at the level of 8-46% (Beumer et al. 2008). Additionally the bacteria may be present on the skin, coat and hoofs (Korsak et al. 1998). The majority of microorganisms are introduced to milk from the contaminated external surface of the teats, equipment or hands of workers (Ayres et al. 1980), although mastitis is also mentioned as the possible contamination source. Lander and Gill (1980) induced experimental infection of the bovine udder with *Campylobacter coli/jejuni* with the subsequent development of a mastitis, and the excretion of this bacteria in milk for several days. The mastitis caused by genus *Campylobacter* bacteria may be severe, acute with loss of appetite and high fever (Gadmundson and Chirino-Trejo 1993). To reduce milk contamination,

the equipment and devices such as milking units, pails, cans and milk churns should be carefully rinsed, cleaned using detergents and disinfected immediately after use (Dodd and Phipps 1994). Assurance of microbiological milk quality is also influenced by the quality of water used for cleaning and rinsing the equipment after disinfection. Additionally, milk quality is also determined by the storage conditions after milking (Aumaitre 1999).

Pasteurization is one of the methods for elimination of pathogens, including *Campylobacter*, from milk (Robinson and Jones 1981) although cases of infection with this pathogen caused by ineffective pasteurization or secondary contamination of pasteurized bottled milk by jackdaws and magpies damaging the caps on bottles with their beaks were also recorded (Hudson et al. 1991). Research conducted by Sockett et al. (1993) indicate that one out of five cases of campylobacteriosis in humans was caused by drinking pasteurized milk from bottles with tops damaged by birds. Also Fahey et al. (1995) described the source of food poisoning with *Campylobacter jejuni* encompassing 110 cases among which 41 represented microbiologically confirmed infection with *Campylobacter jejuni*, caused by consumption of inappropriately pasteurized milk.

The present study showed that all the strains tested were susceptible to four antibiotics: ampicillin, erythromycin, gentamicin and chloramphenicol. In the studies conducted by Sato et al. (2004), none of the isolates tested was also resistant to gentamicin and erythromycin. Also Châtre et al. (2010) did not find the resistance to gentamicin among the isolates of *C. jejuni*. Numerous studies indicate that gentamicin is an effective agent in the treatment of campylobacteriosis in humans (Aarestrup et al. 1997, Li et al. 1998). Also erythromycin is a frequently applied medical drug of choice. The absence of resistance (Sato et al. 2004) or very low resistance to this antibiotic at the level of 1.9% (Chatre et al. 2010), or 2.9% (Bae et al. 2005) suggest that macrolides are one of the first line antibiotics in the treatment of human *C. jejuni* – associated diseases.

In the present study relatively high resistance of the isolated strains to doxycyclin (71.5%) as well as tetracycline and ciprofloxacin (85.8%) was recorded. In the opinion of many authors (Avrain et al. 2003, Ge et al. 2003), the high resistance to tetracycline is frequently recorded in case of genus *Campylobacter* bacteria isolated from products of animal origin. This is confirmed by the studies conducted by Sato et al. (2004), who recorded 45% of isolates resistant to tetracycline. Chatre et al. (2010) also recorded a high level (66.2%) of resistance to this antibiotic.

A very high level of resistance to ciprofloxacin,

reaching up to 85.8%, is a point for consideration in the present study. The studies conducted so far have rather indicated susceptibility of genus *Campylobacter* bacteria to this antibiotic. Bae et al. (2005), Englen et al. (2005) recorded the resistance at the level of 25%, although Endtz et al. (1991) highlighted the increasing resistance of *Campylobacter* species to antibiotics, mainly the fluoroquinolones. As a consequence, fluoroquinolones should not be recommended in the treatment in cases with severe or prolonged symptoms.

According to Keene (1999), raw milk consumption is a high-risk behavior and will continue causing disease and mortality as long as people do not stop consuming raw milk and products made of it.

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