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Highly Resistant *Yersinia enterocolitica* Isolated from Dairy Based Foods in Lebanon

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Abstract

Background: Yersinia is small rod shaped, gram negative coccibacilli known as a food borne pathogen that may cause intestinal and systemic diseases known as yersiniosis. It has been reported that Yersinia might be transmitted by eating contaminated dairy foods.

Methods: This study aims at evaluating the presence of *Yersinia enterocolitica* in three Lebanese dairy based foods which include Kishk, Shankleesh and Baladi cheese and testing their antimicrobial profiles to commonly used antimicrobial agents. Selective media was used to isolate *Y. enterocolitica*, isolates were subjected to relevant biochemical tests and finally identified by API. API confirmed isolates were then tested for their susceptibility to the following antimicrobials: chloramphenicol (30µg), trimethoprim/ sulfamethoxazole (1.25µg+23.75µg), gentamicin (10µg), ciprofloxacin (5µg), nalidixic acid (30µg), Kanamycin (30µg) and streptomycin (10µg).

Results: In total, sixteen *Y. enterocolitica* isolates were identified. Eleven of those isolates were from Baladi cheese, 3 from Shankleesh and 2 from Kishk. Surprisingly, all the tested *Y. enterocolitica* isolates showed high rates of resistance to all the antimicrobials used with highest resistance seen in the case of kanamycin (81.2%) and streptomycin (87.5%). The data showed that the antimicrobial resistance levels exceeded by far all the levels reported elsewhere.

Conclusion: Based on the results, it may be concluded that dairy based foods in Lebanon especially cheese which is not always prepared under proper hygienic practices may become a public health hazard, as it may act as a potential vehicle for the transmission of many resistant bacterial pathogens. For this reason, it is advisable to use strict conditions in cheese and dairy products processing to reduce the hazards that may be involved with its consumption.

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Introduction

Yersinia is small rod shaped, gram negative coccobacilli. This bacterium is heat-sensitive and can be easily destroyed at temperatures of 60°C and higher. Yersinia includes eleven species out of which only *Y. enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis* have been involved in human disease. The other eight species did not show any virulence characteristics inspite of the fact that a large number of them were isolated from sick people, which raises questions concerning their degree of non-pathogenecity of those species (1 and 12).

Being the causative agent of yersiniosis, *Y. enterocolitica* is the most widely studied species of Yersinia (1). *Y. enterocolitica* is a facultative anaerobic microorganism that is motile at 25°C and non-motile at 37°C. This bacterium is not a part

of humans' normal microbiota (28). It grows at temperatures ranging from 0°C to 45°C, with maximum growth observed at temperatures ranging from 30°C to 34°C (28). Environmental isolates of *Y. enterocolitica* do not express their virulence genes in the environment. However, upon entry into the body, pathogenic *Y. enterocolitica* express their virulence markers after encountering the body temperature of 37°C and the low concentration of calcium in the intestinal tract and/or inside the macrophages (11 and 14).

Y. enterocolitica is divided into 70 serotypes among which only few are pathogenic to humans (19). Harmful serotypes are divided into two groups: the American group (O:8, O:13a, O:13b, O:20, and O:21) and the European group (O:3, O:5,27, and O:9) (18). However, serotypes O:3, O:8, O:9, and O:5, 27 are the most common causes for yersiniosis worldwide (11 and 23).

Pathogenic *Y. enterocolitica* carry a variety of pathogenic genes that are either carried by their plasmids or as a part of their chromosomal DNA (6) which in turn encode certain outer membrane proteins that are involved in their pathogenesis. Many studied human pathogenic serotypes harbor a virulent-associated plasmid pYV of 70 to 75 Kbp (3). Among the chromosomally encoded virulence genes *inv*, *ail,* and *yst* are the most important (18). Yersiniosis is characterized by: abdominal pain, diarrhea, and low grade fever. Vomiting occurs in 40% of the cases. In infants, the disease lasts from 3 to 28 days, while in adults it does not usually persist for more than two weeks (4).

Most *Y. enterocolitica* infections occur in children under the age of five. However, infections in adolescents or adults show symptoms similar to those of appendicitis, often resulting in the misdiagnosis of the disease (12). Most yersiniosis cases are self-limiting. However, serious complications may accompany *Y. enterocolitica* infections, especially in immunocompromised individuals. Enterocolitis might occur and it is characterized by the presence of leucocytes in the fecal material. Two percent of patients suffer from reactive arthritis syndromes one to two weeks after the infection. Septicemia might happen in patients with predisposing conditions such as alcoholism and patients suffering from diabetes. In severe cases, when bacteria gets to the mesenteric lymph nodes, an inflammation known as Mesenteric lymphadenitis may result (5).

Y. enterocolitica is known to be the most common cause of bacterial enteritis in Northern and Western Europe. The number of cases of infection detected in North America has decreased over the last few years. *Y. enterocolitica* is transmitted by all kinds of food including milk and dairy products (24).

Between 3,000 and 20,000 cases of *Y. enterocolitica* infections are reported in the USA yearly. Among milk and dairy products related outbreaks, four major ones were reviewed between 1976 and 1995 (29).

More recently, *Y. enterocolitica* was culture-confirmed from 16 patients admitted with symptom onset between March 24 and August 5, 2011 in Pennsylvania. Seven of these patients were hospitalized and three were admitted to the intensive care unit. Investigations showed that all 16 patients had drunk glass-bottled, pasteurized milk from dairy A, three of them were also reported eating dairy A ice cream. When ice cream was tested, one unopened container from the home of a patient with culture-confirmed illness tested positive for *Y. enterocolitica*, as did homemade yogurt made with dairy milk in the home of an asymptomatic person. Both Yersinia cultured from the ice cream and from the homemade yogurt showed matching, as determined using pulse-field gel electrophoresis, patterns of genomic DNA patterns with those isolated from the stool samples of nine patients (8).

In addition to milk and dairy products, which are the interests in this study, pigs serve as a major reservoir for *Y. enterocolitica*. The organism may be contracted by humans during swine slaughtering and mainly throughout the evisceration process there is an increased chance for the contamination of pork carcass with potentially pathogenic bacteria such as *Y. enterocolitica* and *Salmonella* if proper hygienic practices are not followed. A recent study in Bavaria, Germany showed that 81 out of 446 samples of pork products were contaminated with *Y. enterocolitica* (21).

Materials and Methods

Samples Collectios

Baladi cheese, shankleesh and kishk were collected from the Bekaa valley area of north-east Lebanon. Samples were collected on 4 trips between the months of August and December 2004. Target locations for sample collection included houses, markets, and small family farms. In total, 164 samples were collected (83 kishk, 45 baladi cheese and 36 shankleesh). All samples were packaged in sterile bags and kept on ice in a refrigerator until brought to the laboratory. Samples were analyzed within 24 hours.

Bacterial Isolation and Enumeration

Microbiological analysis was conducted on 25g portion of each food sample placed aseptically in sterile stomacher bag (Seward Medical Stomacher Bags © Seward, Germany). Two

hundred and twenty five mls of sterile 1% peptone water (Hi media laboratories limited, India) were added to the sample and the contents were macerated in a stomacher (Seward, Germany) for 3 minutes. Extreme precautions were taken throughout the procedure to avoid contamination. Serial dilutions of the homogenate were prepared (10^{-1} - 10^{-3}) using sterilized peptone water (13). A duplicate of each dilution was inoculated into Cefsulodin-Irgasan-Novobiocin (CIN) plates (Oxoid, Basinstoke, England) that were used for the selection of *Y. enterocolitica* (33).

Bacteriological analyses were performed according to the Compendium of Methods for the Microbiological Examination of Foods (9) and Official Methods of Analysis of AOAC International (15), by innoculating in duplicates with 0.1 ml of each dilution agar plates containing the appropriate selective media. Plates were incubated at 32°C for 48 hours.

Colonies were counted and the CFU/g for each sample was determined. Colonies with suspected colors and morphologies were simultaneously patched on clean plates and cultured in 5 ml of BHI broth. Plates and tubes were incubated at 37°C for 24 hours. Finally, the plates were stored at 4°C, whereas 500µl of the broth culture were mixed with 500µl of 50% sterile glycerol in freezing tubes and stored at –70°C to preserve bacteria for later usage (31).

Bacterial Identification using Biochemical Tests

Y. enterocolitica colonies are characterized by their unique bull's-eye morphology on the CIN agar (1.5 mm diameter, deep red/purple center with a sharp edge surrounded by a translucent border). Therefore, only colonies with suspected morphologies were selected and tested by gram staining (10 and 26). Suspected colonies were then confirmed using the API 20E biochemical system (bio Merieux, Marcy l'étoile, France). Positively confirmed isolates were later molecularly characterized using PCR (22).

DNA Extraction

DNA of *Y. enterocolitica* suspected colonies was extracted using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, UK) as described by Saleh *et al* (27).

Primers Design and PCR

Molecular characteristics of suspected colonies were investigated using four sets of primers, three of which were selected based on specific virulence genes in pathogenic *Y. enterocolitica*, while the remaining set was designed to identify the *Y. enterocolitica* O: 3 serogroup (Table 1). PCR test was conducted as described elsewhere (27). Ten-µl of each PCR product were then mixed with 2µl of loading dye (6X) (Biorad, USA) and run on a 1.5% agarose gel containing 0.25µg per ml of ethidium bromide. Gels were visualized under a UV illuminator and photographed (13).

Table 1. Sets of primers used in *Y. enterocolitica* identification and their target genes

Identified Bacteria	Primers name	Amplified Gene(s)	Reference
Pathogenic Y. enterocolitica	Ail F – Ail R	Attachment invasion locus	3
	Pr2a – Pr2c	Heat stable enterotoxin	16
	YE-1 – YE-2	Enterotoxin gene	35
O:3 serogroup	Rfbc F - Rfbc	O side chain	36

Antimicrobial resistance test

Yersinia enterocolitica characterized strains were tested for their susceptibility to different antimicrobials, using the disk diffusion method as set by the National Committee for Clinical Laboratory standards (2). Organisms were cultured in 5 ml BHI broth and grown in a shaking water bath at 37 °C overnight. Then, 0.1 ml of each culture were inoculated onto Mueller Hinton agar plates (Oxoid, Basinstoke, England) and disks impregnated with different antimicrobials (BioMerieux, France) were placed on the plates to determine the extent of inhibition after appropriate incubation. Seven antimicrobials were used: chloramphenicol (30µg), trimethoprim/ sulfamethoxazole (1.25µg+23.75µg), gentamicin (10µg), ciprofloxacin (5µg), nalidixic acid (30µg), Kanamycin (30µg) and streptomycin(10µg) (7 and 20). Zones of inhibition around each antimicrobial disk were measured after incubation at 37°C for 24 h. Using NCCLS guidelines, organisms were classified as: resistant (not inhibited), intermediately resistant (not completely inhibited), or susceptible (inhibited) to the antimicrobials

Results

Y. enterocolitica Count and Identification using Biochemical Tests

Sixteen bull's-eye colonies were identified on CIN media and therefore were suspected to be *Y. enterocolitica.* Gram staining showed that all suspected colonies are negative rods. Finally, API test had confirmed all of the sixteen isolates as *Y. enterocolitica*.

Contamination Levels of the Three Tested Dairy Based Foods

Eleven out of the sixteen identified bacteria were isolated from cheese samples, which means that 24.4% of the tested cheese samples are contaminated with *Y. enterocolitica*. As for Shakleesh and Kishk samples, 8.3% (3 samples) and 2.4% (2 samples) of the tested sample are respectively contaminated. Overall, 164 dairy based food samples were tested, 9.7% are proved to be contaminated.

Characterization of the Pathogenic *Y. enterocolitica* by PCR

None of the 16 isolates has shown any of the tested genes.

Antimicrobial Susceptibility of the Suspected *Y. enterocolitica* Isolates

The 16 colonies biochemically identified as *Y. enterocolitica* were tested for their susceptibility to 7 different antimicrobials. Isolates showed the highest resistance rate to streptomycin with 87,5% resistance, while the lowest rate was shown with nalidixic acid (31.2%) (**Figure 1**).

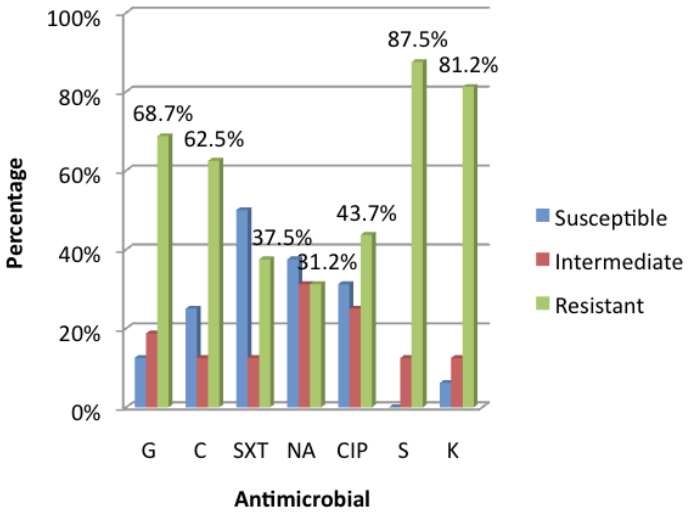

Fig. 1. The percentage of antimicrobial resistance patterns of the 16 *Y. enterocolitica* isolates. The antimicrobial codes are: G: Gentamicin; C: Chloramphenicol; SXT: Trimethoprim-sulfamethoxazole; NA: Nalidixic acid; CIP: Ciprofloxacin; S: streptomycin; K: Kanamycin.

Discussion

This is a novel study on the presence of *Y. enterocolitica* in dairy based foods in Lebanon. Few studies were conducted on the presence of this bacterium in milk samples worldwide. Old studies conducted on raw milk in Alsace, France showed that out of 75 tested samples, 61 (81.4%) were contaminated with *Yersinia spp*. (34). In Ireland, 589 samples were studied out of which 279 tested positive for *Yersinia spp*. Fifty nine percent of those were *Y. enterocolitica*. (25). In Turkey, a study performed on 211 raw milk samples revealed 33 pathogenic microorganisms, among which 8 were *Y. enterocolitica* (32). In Northern Iran in 2003, a study on raw milk samples, showed that 1.6% of all tested samples (120 samples) were contaminated with *Y. enterocolitica*. No *Y. enterocolitica* were isolated from any of the 40 tested pasteurized milk samples (30). In Pennsylvania, 248 samples of bulk tank milk were tested, 1.2% of which showed contamination with *Y. enterocolitica* (17). In this study, 9.75% of the samples tested positive for *Y. enterocolitica* and no pathogenic *Y. enterocolitica* would be detected. However, the degree of non-pathogenecity of our isolates needs further investigation. The first set of primers used to identify pathogenic serogroups among the isolated *Y. enterocolitica* was designed to identify all pathogenic strains of *Y. enterocolitica* regardless of the bioserogroup. The design of those primers was based on what is known about the sequences of the most common virulent strains of *Y. enterocolitica,* which are divided into two broad groups commonly referred to as the American or the European varieties (16). The classification of *Yersinia* isolates as non-pathogenic is based on the absence of classical *Yersinia* virulence markers based on the two varieties mentioned earlier. However, the isolation of high numbers of *Yersinia* with no known virulence markers from persons with yersiniosis has raised the question of their possible pathogenecity (12). *Y. enterocolitica* is a major food-borne pathogen, it is one of the most important bacteriological causes of diarrhea in Germany. However, studies on the occurrence of human pathogenic *Y. enterocolitica* in food are very rare (21) and studies concerning the molecular characterization of *Yersinia* isolates were mainly concentrated on the American and the European isolates and those may not include the species that are present in the Middle East. More studies need to be performed in order to better understand the virulence characteristics of strains in Lebanon and the Middle East.

Yersinia enterocolitica is known worldwide that it does not easily acquire antimicrobial resistance. Studies done in the developing countries showed that *Y. enterocolitica* strains are susceptible to the majority of commonly used antimicrobials. The results obtained in our study have exceeded all expected levels and showed, surprisingly, that *Y. enterocolitica* isolated from dairy-based foods were highly resistant to most antimicrobials. The *Y. enterocolitica* isolated from Lebanese dairy foods showed high rates of resistance to gentamicin, kanamycin, nalidixic acid, trimethoprim/ sulfamethoxazole, chloramphenicol and sterptomycin. This is in contrast to results obtained from a study conducted in Austria on the antimicrobial pattern of bacterial resistance isolated from different meat products (pork, beef, chicken and turkey) that showed 100% susceptible to all those antimicrobials (20). This is probably because Austria, as a developed country, has strict policies are imposed on the use of antimicrobials.

Conclusion

The results are quite alarming and emphasis the need of usage of proper hygienic practices in the preparation of dairy based foods. This is addition to the need for policies to restrict the use of antimicrobials in the food chain and for therapeutic purposes. Education of people on the health hazards associated with the use of antimicrobials should be emphasized and the role of the appropriate governmental agencies should practise their role in controlling the use of antimicrobials.

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