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Note

Resistance of *Brucella abortus* isolated from Lebanese dairy-based food products against commonly used antimicrobials

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Abstract – Considering the marked importance of *Brucella* organisms as food-borne pathogens and the lack of published literature on the evaluation of the microbiological quality of dairy-based food products in the Middle East, this study was performed to address this gap. The main aim of the present study was to assess the antimicrobial resistance patterns of *Brucella* isolates recovered from a total of 164 cultured samples of Lebanese dairy-based food products (Baladi cheese, Shankleesh and Kishk). Standard polymerase chain reaction (PCR) was used to stereotype colonies previously confirmed by biochemical tests to be *Brucella* strains and to distinguish between the RB51 vaccine and field *Brucella* strains. Real-time PCR was applied to differentiate among the various *Brucella* species. Confirmed PCR field *Brucella abortus* isolates were evaluated for their susceptibility to eight commonly used antimicrobials. The highest number of resistant *B. abortus* isolates (n = 4 out of 6) was shown against Streptomycin and Ciprofloxacin, whereas 3 out of 6 isolates tested were resistant to Gentamicin. A lower number of resistant isolates were noted against Rifampicin, Tetracycline and Trimethoprim-sulfamethoxazole (n = 2 out of 6) and the lowest number for Doxycycline and Ceftriaxone (n = 1 out of 6). Such results are alarming and reflect the significance and importance of implementing more strict hygiene standards and regulations to reduce food-borne illnesses and control the excessive use of antimicrobials in this region.

***Brucella abortus* / dairy product / antimicrobial resistance / real-time PCR**

摘要 – 源于乳基食品的 *Brucella abortus* 对常用抗菌药物的耐药性。摘要 布鲁氏菌是一种很常见的食源致病菌，以前没有关于中东地区乳基食品微生物品质方面的研究报道，本研究的开展将填补这一空白。 本研究评价了从 164 个源于黎巴嫩乳基食品样品中 (包括 Baladi、 Shankleesh 和 Kishk 干酪) 分离得到的布鲁氏菌的耐药模式。 利用标准聚合酶链式反应 (PCR) 对以前用生化方法检验过的菌株进行验证以区分 RB51 疫苗和布鲁氏菌株。 以实时 PCR 技术区分不同布鲁氏菌属。对经 PCR 反应确认后的 *Brucella abortus* 菌株进行 8 种常用抗菌剂的耐药性研究。 结果表明耐链霉素和环丙沙星的 *B. abortus* 菌株最多 (6 株中有 4 株) ; 3 株能耐庆大霉素； 耐利福平、四环素、甲氧苄啶、磺胺甲恶唑的菌株就

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更少了 (6 株中有 2 株)；最低的是耐强力霉素和头孢三嗪的菌株 (6 株中只有 1 株)。 这个结果提示我们，在这一地区如果要减少食源性疾病和控制过度使用抗菌药物，必须实施更加严格的卫生标准和制度。

***Brucella abortus* 菌 / 乳基食品 / 耐药性 / 实时聚合酶链式反应 (PCR)**

Résumé – Résistance de *Brucella abortus* isolée de produits alimentaires libanais à base de lait contre les antibiotiques utilisés couramment. Cette étude a été menée afin de combler le manque de publication portant sur l'évaluation de la qualité microbiologique des produits alimentaires à base de lait au Moyen Orient, au vu de l'importance marquée des *Brucella* comme pathogènes d'origine alimentaire. Le principal but de cette étude était d'établir les profils de résistance aux antibiotiques des isolats de *Brucella* obtenus à partir de 164 échantillons de produits alimentaires libanais à base de lait mis en culture (fromage Baladi, Shankleesh et Kishk). La PCR standard a été utilisée pour stéréotyper les colonies préalablement confirmées par des tests biochimiques comme des souches de *Brucella*, et pour différencier le vaccin RB51 des souches de *Brucella* de terrain. La PCR en temps réel a été appliquée pour distinguer les différentes espèces de *Brucella*. La susceptibilité aux antibiotiques des isolats confirmés de *Brucella abortus* de terrain a été évaluée pour huit antibiotiques couramment utilisés. Le nombre le plus élevé d'isolats de *B. abortus* (n = 4 sur 6) était résistant envers la streptomycine et la cyclofloxacine, alors que 3 des 6 isolats testés étaient résistants à la gentamicine. Un nombre plus faible d'isolats était résistant à la rifampicine, la tétracycline et le trimethoprim-sulfamethoxazole (n = 2 sur 6) et le plus petit nombre (n = 1 sur 6) était résistant à la doxycycline et la ceftriaxone. De tels résultats sont alarmants et reflètent l'importance de mettre en œuvre des normes d'hygiène et des réglementations plus strictes pour réduire les maladies d'origine alimentaire et contrôler l'utilisation excessive des antibiotiques dans cette région.

***Brucella abortus* / produit à base de lait / résistance aux antibiotiques / PCR en temps réel**

1. INTRODUCTION

The consumption of dairy-based foods may play an important role in the transmission of various food-borne diseases. Among those diseases is brucellosis, which is considered by the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (OIE), as one of the most widespread zoonotic diseases of domestic and wild animals throughout the world [21, 23]. Brucellosis is a "re-emerging" disease leading to substantial economic loss as well as considerable human morbidity [21]. It is a disease caused by several species of the genus *Brucella* [25].

Based on host preferences and pathogenicity, the genus *Brucella* has been divided into six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*. Out of these six species, only *B. melitensis* and *B. abortus* are the major pathogenic species in human beings and animals. *B. melitensis* is typically pathogenic in goats and sheep, while *B. abortus* is pathogenic in cattle [25]. Brucellosis can be transmitted to human beings by direct contact with materials contaminated with the organism, consumption of unpasteurized milk and milk products, and inhalation of infectious aerosols, especially by workers in abattoirs and microbiology laboratories [22, 25]. The most common signs and symptoms of this disease are undulant fever, weakness, chills, headache, depression and weight loss which can persist from days to years [23].

Traditionally, bacterial isolation and biochemical identification of most pathogenic organisms are slow, laborious and insensitive. Therefore, molecular genetic techniques have recently been involved

in studies regarding taxonomy and evolution of bacteria. One of these powerful techniques is PCR, which has become a promising tool for the identification and characterization of bacteria such as *Brucella* to genus and species level in food products such as milk and cheese [3, 4, 16].

Contaminated and unpasteurized dairy products have been responsible for outbreaks of many food-borne diseases [6]. In Lebanon, dairy products made of unpasteurized milk are usually consumed raw by a large group of the population on a daily basis. While some of those products are prepared in dairy plants, many are still homemade. Dairy food production in Lebanon mostly occurs in its northeastern part, mainly in the Bekaa Valley.

In the last five years, there has been a noticeable increase in the number of food-borne diseases in Lebanon where about 657 cases were attributed to food-borne illnesses in 2005 [9]. This reported increase has highlighted the need to implement control measures in the food industry and the importance of studies that evaluate the microbiological quality of foods. Considering the marked importance of *Brucella* as a food-borne pathogen, this study aims at isolating and characterizing at the molecular level the different strains of *Brucella* present in dairy products which are consumed raw in Lebanon. The study also evaluates the antimicrobial resistance patterns of *B. abortus* to eight different commonly used antimicrobials. The foods included in this study are Baladi cheese (Lebanese cheese balls), Shankleesh (a mold-ripened cheese) and Kishk (a dried fermented milk-wheat mixture).

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 164 samples of Baladi cheese (n = 45), Shankleesh (n = 36) and Kishk (n = 83) were collected from the Bekaa Valley in four field visits conducted from August till December 2004. Samples were obtained from different sources (such as markets, houses and small family dairy farms), packaged in sterile bags, numbered and brought to the laboratory on ice in an ice chest. All samples were analyzed within a maximum of 24 h after their arrival to the laboratory [20].

Samples were collected from different communes, which were divided into four categories based on their number of inhabitants, with samples collected from 20% to 100% of the communes in each category (Tab. I). Most of the samples taken from the small communes were homemade. The original plan was to collect around 250 samples, noting that the number of samples collected in each commune should be roughly proportional to the size of the commune. However, many of the small communes selected for study could not be visited because of bad weather conditions which made access to some roads impossible. In addition, homemade dairy products were not made on a daily basis and sometimes not during our collection trip, which decreases the total number of samples collected to 164 samples divided into 83 Kishk samples, 45 Baladi cheese samples and 36 Shankleesh samples.

2.2. Bacterial isolation and/or enumeration

The bacteria examined in the dairy-based food products included indicator bacteria (aerobic plate count and total coliforms) which were published in a previous study by our group [20] and pathogenic *Brucella* organisms. For the detection, enrichment and plating of *Brucella* colonies, a selective medium *Brucella* Agar (Difco, Paris, France) was used [18]. Bacterial isolation, enumeration and analyses were done according to Harakeh et al. [13], with the

Table I. Number of selected communes according to population size and the number of samples that should be collected in each trip.

Commune type	No. of inhabitants	No. of communes	Population size	Population size (%)	Selected commune	No. of visited communes per trip	No. of samples collected per trip
A	< 1000	181	67 730	19.6	36	3–4	3–4
B	1000–5000	75	163 300	47.1	34	3–4	9–12
C	5000–10 000	5	31 000	9.0	5	3	3
D	> 10 000	4	84 000	24.3	4	3	6
Total		265	346 030	100	79		

exception that all plated cultures were incubated at 37 °C for 48–72 h [8, 14, 15, 19].

It is worth noting that one of the *Brucella* species, *B. abortus*, requires microaerophilic conditions of 5–10% CO_2 for enhanced growth. Thus, plates were divided into two sets, incubated either under aerobic conditions or in a microaerophilic environment containing 5–10% CO_2 conditions [1, 18]. Colonies that exhibited *Brucella* morphology were counted and preserved for further analyses.

2.3. Biochemical identification of the suspected *Brucella* species

Suspected *Brucella* colonies that are characterized by punctate, raised, circular, translucent and white mucoid opaque appearance were first identified by Gram staining [1]. For further confirmation, Gram-negative coccobacilli were simultaneously subjected to oxidase (Kovac's modification) and urea hydrolysis (Christensen's method) tests [5].

2.4. DNA extraction

Using a sterile loop, a bacterial colony was suspended in 5 mL of sterile Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, UK). This suspension was incubated overnight using a shaking water bath at 37 °C until a 0.5 McFarland turbidity standard was reached. DNA extraction was conducted using 1 mL of the bacterial suspension to extract total genomic DNA according to the GFX genomic blood DNA Purification Kit from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) [13].

2.5. Primers used

For the genus-specific identification of *Brucella*, standard PCR was used where extracted DNA of the suspected *Brucella* isolates was amplified using the primer pair BRU-UP/BRU-LOW. This primer pair targets a 443 base pair (bp) fragment of the *BCSP-31* gene, encoding for an antigen localized on or near the bacterial cell surface of *Brucella* species [23]. Then, real-time PCR was used to distinguish among the different *Brucella* species. The forward primer used was obtained from the insertion element *IS711*, whereas the reverse primers were derived from species-specific chromosomal loci [19]. The former generates a 113 bp PCR product with *B. abortus* reverse primer, a 252 bp PCR product with *B. melitensis* reverse primer and 170 bp PCR products with *B. suis* reverse primer. To generate the fluorescence signal, a double-stranded DNA intercalating dye, SYBR Green I, was used [19].

Finally, the primer pair (1 and 2) was used to determine whether the *B. abortus*

Table II. Applied PCR cycles for the detection of genus- and species-specific *Brucella* and differentiation between vaccine and field strains.

	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Post-PCR step
Genus-specific identification	95 °C for 5 min	40	94 °C for 30 s	56 °C for 30 s	72 °C for 1 min	72 °C for 7 min
Differentiation of *Brucella* species	95 °C for 35 s	45	95 °C for 30 s	58 °C for 30 s	72 °C for 45 s	72 °C for 10 min
Differentiation between vaccine and field strains	–	40	95 °C for 1 min	62 °C for 1 min	72 °C for 1.5 min	–

isolates were derived from RB51 vaccine or field strains as stated in Vemulapalli et al. [24]. If the *B. abortus* isolates were RB51 vaccine strains, then the PCR product would amplify a 1300 bp band indicating that the *wboA* gene is interrupted by an *IS711* element. However, a 400 bp fragment would be produced from all other *Brucella* species and strains, revealing that the *wboA* gene remained intact [24].

2.6. PCRs

Standard PCR was performed in a total volume of 25 µL reaction mixture containing: 1.25 µL of each primer (10 pmol·µL^{-1}), 3 µL of 50 ng·µL^{-1} of the purified DNA template, 2.5 µL of 10 X PCR buffer (AB-gene products, Epsom, UK), 1.875 µL of $MgCl_2$ (25 mmol·L^{-1}) (AB-gene products, Epsom, UK), 0.5 µL of each dNTP (100 mmol·L^{-1}) (dATP, dGTP, dCTP and dTTP) (AB-gene products, Epsom, UK) and 0.2 µL of 5 U·µL^{-1} of *Thermus aquaticus* (Taq) DNA polymerase (AB-gene products, Epsom, UK) [14]. The volume was brought up to 25 µL by adding sterile double distilled water. The mixture was placed in a thermocycler, Icycler (Bio-Rad, Hercules, California, USA). A negative control (no DNA template) and a positive control of *B. melitensis* DNA template, obtained from the American Type Culture Collection via the American University of Beirut Medical Center, Beirut, Lebanon, were included in every PCR assay. The cycles used for the amplification of the targeted *BCSP-31* are summarized in Table II [22].

For real-time PCR, a typical 25 µL reaction contained 0.5 µL of 200 nmol·L^{-1} of each primer, 2 µL of 50 ng·µL^{-1} of DNA template and 12.5 µL of 1 X IQ SYBR Green Supermix (100 mmol·L^{-1} KCl, 40 mmol·L^{-1} Tris-HCl, pH 8.4, 0.4 mmol·L^{-1} of each dNTP (dATP, dCTP, dGTP and dTTP), 50 U·mL^{-1} Taq DNA polymerase, 6 mmol·L^{-1} $MgCl_2$, SYBR Green I, 20 nmol·L^{-1} fluorescein and stabilizers) (Bio-Rad, Hercules, California, USA). The volume was brought up to 25 µL by adding sterile double distilled water. The above mixtures were loaded into the wells of a 96-well plate and amplification was performed using a thermocycler (Bio-Rad, Hercules, California, USA). The amplification procedure is summarized in Table II [19].

The same standard PCR assay, used for the genus-specific identification, was performed for the amplification of the *wboA* gene. Table II also shows the applied cycles used for the differentiation between field and vaccine strains [24]. All the reactions (in both standard and real-time PCR) were terminated after 1 h incubation at 4 °C.

2.7. Agarose gel electrophoresis

Ten-microliter aliquot of each of the PCR products was mixed with 2 μL of 6 X loading dye (Bio-Rad, Hercules, California, USA). The PCR products were electrophoresed on 1% agarose gel at 90 V for 90 min. An EZ load 100 bp ruler (Bio-Rad, Hercules, California, USA) was used as a DNA ladder. After the run was completed, the bands were visualized under UV light and photographed.

2.8. Antimicrobial susceptibility testing

PCR-confirmed *Brucella* isolates were tested for their susceptibility to different antimicrobials, using the disk diffusion method as set by the National Committee for Clinical Laboratory Standards [2, 17]. The same protocol used in Harakeh et al. [12] was applied here for the antimicrobial susceptibility testing of *Brucella*.

The following antimicrobial disks, obtained from BioMerieux (Craponne, France), were used: Doxycycline (30 mcg), Streptomycin (10 mcg), Gentamicin (10 mcg), Tetracycline (30 mcg), Rifampicin (30 mcg), Ciprofloxacin (5 mcg), Ceftriaxone (30 mcg) and Trimethoprim-sulfamethoxazole (1.25 + 23.75 mcg) [1].

3. RESULTS AND DISCUSSION

3.1. Levels of contamination with suspected *Brucella* and identification of *Brucella* species using conventional and molecular characterization methods

The levels of contamination with suspected *Brucella* species grown under either aerobic or microaerophilic conditions were highest in Shankleesh (16.7%), followed by Baladi cheese (13.3%) and Kishk (4.8%). It is documented that the presence of any *Brucella* organism in food products is unacceptable and can result in health-related problems [3, 11]. Based on this, about 13.3% of Baladi cheese, 16.7% of Shankleesh and 4.8% of Kishk samples were considered unacceptable for human consumption.

The suspected *Brucella* colonies (n = 110) displaying translucent, white and mucoid appearance were Gram stained. All the suspected isolates were Gram-negative coccobacilli. Only 8 out of the 110 were found to be oxidase positive by showing a light to dark blue color and urease positive by giving a pink color. Based on the data, the eight colonies could be *B. melitensis*, *B. abortus*, *B. suis* or *B. canis*.

For further characterization, the eight isolates were analyzed by PCR for the *BCSP-31* gene using the primer pair BRU-UP and BRU-LOW. Based on the PCR data, it was found that 6 out of the 8 suspected isolates (75%) belonged to *Brucella* species with a characteristic band at ~ 443 bp. Real-time PCR was performed on the confirmed isolates in order to determine the different species of *Brucella*. The same forward primer was used in all the PCRs along with the three selected respective reverse primers specific for *B. melitensis*, *B. abortus* and *B. suis*. Only positive isolates with the *B. abortus* primer pair showed an increase in fluorescence, thus indicating amplification. The results indicated that all six isolates were *B. abortus* strains showing a specific PCR product of about 113 bp on agarose gel (Fig. 1). To determine whether the confirmed *B. abortus* isolates were RB51 vaccine or field *B. abortus* strains, standard PCR was used. The results indicated that all *B. abortus* isolates were field and not RB51 strains showing a 400 bp and not a 1300 bp fragment as is the case in vaccine strains [24]. Based on the PCR data, *B. abortus* was detected in approximately 4% of Baladi cheese, 6% of Shankleesh and 2% of Kishk samples,

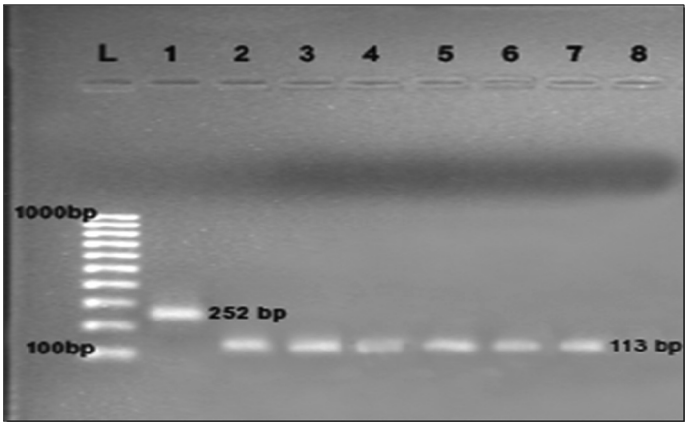

Figure 1. The electrophoretic profile of the real-time PCR products with the primer pair used for the detection of *B. abortus*, except for the positive strain. L: DNA ladder (EZ load 100 bp ruler); 1: *B. melitensis* positive strain; 2–7: DNA of suspected *Brucella* colonies among which two were isolated from cheese samples (Lanes: 3 and 6 (grown under microaerophilic conditions)), two from Shankleesh (Lanes: 2 (grown under microaerophilic conditions) and 5) and two from Kishk samples (Lanes: 4 and 7); 8: negative control.

respectively. Such levels could be due to the absence of eradication of brucellosis in cows in Lebanon.

These percentages of positive samples could be due to poor sanitary standards related to several factors. These factors involve direct contamination from animals via intra-mammary secretions or via fecal contamination of the udder, usage of same milking machine on many cows without frequent and proper cleaning, usage of non-cleaned milk bulk tanks, storage of milk at inappropriate temperatures and improper handling and transportation of milk. In addition to that, lack of hygienic practices during the processing of these products may also increase the contamination levels.

3.2. Antimicrobial susceptibility of *B. abortus* isolates

Brucella abortus isolates ($n = 6$) were resistant to at least one of the tested antimicrobials. High resistance ($n = 4$) was noted against Streptomycin and Ciprofloxacin, while 3 out of 6 isolates showed resistance to Gentamicin. Lower resistance was seen in response to Rifampicin, Tetracycline and Trimethoprim-sulfamethoxazole. High susceptibility was observed against Ceftriaxone and Doxycycline (Fig. 2). It is important to mention that these data are preliminary due to the low number of isolates. More future samples need to be collected and more isolates will be tested for their antimicrobial susceptibility. However, such findings are alarming and indicative of potential emergence of resistance strains of *Brucella* to antimicrobials. This emergence of antibiotic-resistant strains should be of great concern to the public, especially dairy producers, their families and employees because this organism is resistant to antimicrobials that are commonly used in medical treatment of human beings and in veterinary practices. The emergence of resistant bacterial strains might be attributed to the indiscriminate and uncontrolled use of antimicrobials to control diseases in infected herds of Lebanon.

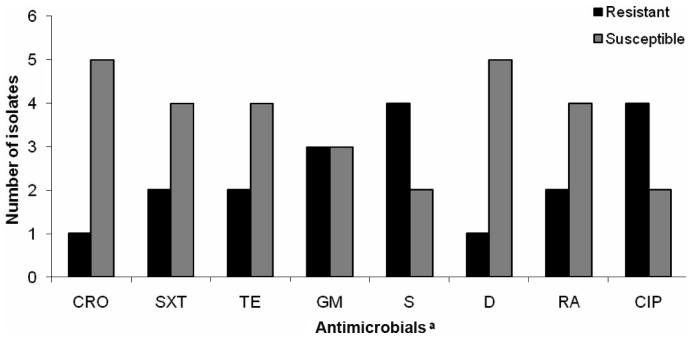

Figure 2. Antimicrobial resistance patterns of the six *B. abortus* isolates. [a]CRO = Ceftriaxone; SXT = Trimethoprim-sulfamethoxazole; TE = Tetracycline; GM = Gentamicin; S = Streptomycin; D = Doxycycline; RA = Rifampicin; and CIP = Ciprofloxacin.

As stated in the literature, this resistance could be due to various mutations in chromosomal genes and not plasmid mediated [2, 14]. Moreover, Gram-negative bacteria have the ability to acquire resistance to a wide range of antimicrobials through the activation of drug efflux pumps. One type of efflux pump, the Resistance Nodulation Division (RND), is responsible for resistance phenotype observed in many bacterial species against a wide range of antimicrobials such as Quinolones, Tetracycline, Chloramphenicol, Streptomycin, Ampicillin, Rifampicin and others. A recent study conducted by Fernando et al. [10] has proved the involvement of two RND systems in mediating antimicrobial resistance in *B. suis*. Further studies are required to prove the role of such mechanism in the antimicrobial drug resistance detected in our samples [7, 10].

4. CONCLUSION

The results of this study provide an important baseline for the contamination status of Lebanese dairy-based food products by *B. abortus* and the patterns of its resistance to commonly used antimicrobials. The presence of multi-drug resistance strains is alarming. Obviously, the implementation of quality assurance programs such as HACCP in the preparation and processing of these foods and the control of usage of antimicrobial agents would help in reducing the risks of infections and the emergence of drug-resistant bacteria.

In addition, public education is required to increase people awareness to the dangers associated with the consumption of ready-to-eat foods that are not cooked or heated enough including unpasteurized milk, especially if consumers are immunocompromised people such as pregnant women, elderly or persons on immunosuppressive drug therapy. Moreover, additional research is required to better understand the mechanisms involved in the spread of antimicrobial resistance among environmental bacterial isolates and to determine the factors and reasons behind food contamination.

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