Molecular Characterization of Virulence Factors and Microbial Resistance of Different Bacterial Isolates in some Dairy Products

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Research article

Keywords: resistance, gene, cheese, bacteria, dairy products

Posted Date: February 25th, 2020

DOI: https://doi.org/10.21203/rs.2.24496/v1

Abstract

Background: Bacterial contamination of milk and dairy products is a common problem. Foodborne microbial diseases reason for 20 million cases annually in the world. In the last two years, foodborne diseases caused by the intake of dairy products have been mostly disturbed with *Salmonella entertica, Listeria monocytogenes Escherichia coli* 0157:H7 and *Campylobacter jejune.* Aim of the study is to isolate MDR bacteria in dairy product and study of molecular characterization of that isolates.

Results: A total 30 out of 131 bacterial isolates were MDR and distributed as 50 % from white cheese, 36.7% from industrial white cheese, 13.3 from old cheese and 6.6 % roomy cheese. The incidence of MDR bacterial isolates revealed the abundance of *Staphylococcus* sp. with 43.3% among all the tested bacterial isolates, while the other tested isolates showed *Bacillus* sp 16.7%, *Salmonella* 13.3%, *E.coli* 10 %, *Enterococcus* 6.7 % *Psedoumonas* 3.3 %, *Shegella* 3.3 % and *Proteous* 3.3 %. Molecular studies of genes presence or absence for class A (TEM, CTX and BSHV), class B (VIM, IMP, KPC and NDM), class C (FOX) and class D (OXA-10, OXA-24 and OXA-58) were tested. NDM, TEM, CITM and (OXA -10) genes were the most abundant the selected bacterial isolates.

Conclusions: The results of this study indicate that cheese made from unpasteurized milk can pose a significant risk to consumers. Product manufacturing processes should be subject to health control-to-control pathogens. Reducing the surface area exposed to air reduces harmful microbial growth in dairy products.

1. Background

Spoiling milk products worldwide is a huge economic problem. The microbial capacity and incidence of the bacterial pathogens in foods are indicators of food quality. In addition, the education of food handlers about personal hygiene is importance from food safety point of view [1]. The highly nutritious nature of dairy products makes them especially good media for the growth of microorganisms [2]. The microbial contamination is one of the leading causes of food spoilage worldwide [3]. The contamination of food with microbes can occur at any stage of the foodchain[4].A large number of diseases are caused by foodborne pathogens with significant effects on economy and human health.Bacterial pathogens use a variety of different motility modes, including swimming, twitching, and swarming [1]. S.aureus is commonly food born pathogen of great importance of animal and human concern. it is responsible for contaminate dairy products, kariesh cheese and ice cream from different sources during their production, processing and handling that make them unfit for human consumption or even a dangerous source of infection among customers establishing a potential health hazard [5]. S. aureus is also known for its ability to secrete a host of toxins to aid in host tissue infiltration and acquire nutrients [6]. The features of the most common pathogenic bacteria (Salmonela, Shigella, Listeriamonocytogenes, Bacilluscereus, Campylobacter, Clostridium, Cronobacter, E.coli, Staphylococccus aureus, Vibrio, Yersinia enterocolitica, viruses (Hepatitis A and Noroviruses) and parasites (Cyclospora cayetanensis, Toxoplasma gondii and Trichinella spiralis [7, 8]. Antimicrobial resistance (AMR): the ability of microbes to grow in the presence of a drug that would normally kill them or limit their growth [9, 10]. AMR complicates infection treatment is linked to increased mortality and morbidity. The emergence and spread of resistant and multidrug-resistant (MDR) bacteria has enormous implications for worldwide healthcare delivery and population health [11, 12, 13].Virulence functions are often encoded on large extrachromosomal plasmids by pathogenic bacteria. These plasmids are maintained at low copy number to reduce the metabolic burden on their host [14, 15]. The widespread use of extended-spectrum cephalosporin creates a reservoir of resistant bacteria. Moreover, multi-resistance frequently associated with strains carrying ESBLs, which could dramatically reduce the treatment options. The increasing number of Enterobacteriaceae with ESBLs that also contain MBLs or AmpCs and other new mechanisms of resistance to fluoroquinolones or aminoglycosides indicate that recent increase of ESBLs –producing bacteria in Europe constitutes a complex problem [16]. ESBLs are worthy of the scientific community's attention over the past decades among the β-lactamases. ESBL's older and classical definition includes TEM-1, TEM-2, or SHV derivatives. ESBL is divided into three main groups by the most recent definition. (I) ESBLA (class A ESBLs): CTX-M, SHV and TEM enzymes. (II) ESBLM (miscellaneous ESBLs) are sectioned into ESBLM-C (class C, plasmid mediated AmpC) and ESBLM-D (class D). (III) ESBLCARBA (ESBLs that degrade carbapenems) are divided into ESBLCARBA-A, ESBLCARBA-B, and ESBLCARBA-D. More than 500 β-lactamases have been reported to date produced by diverse bacteria. Beta-lactamases be the most common resistance mechanism that contributes to widespread resistance among Gram-negative microbes [17]. Transmission of resistance occur between microorganisms [18]. NDM-1 producing E. coli infects the host by commonly invading sites like, urinary tract, blood, lungs, and wounds, leading to urinary tract infections, septicemia, pulmonary infections, diarrhea, peritonitis, device-associated infections and soft tissue infections [19].

2. Methods

2.1. Samples

Dairy products: samples from different locations in Alexandria were collected during 2018: white cheese, white cheese produced, old cheese and milk. The samples were collected for further use in sterile containers.

2.2. Assessment of isolated bacteria's resistance prevalence

According to the modified Kirby-Bauer Disc Diffusion method, all the isolated bacteria (130 isolates) were subjected to antibiotic resistance using the disk diffusion method.

2.3. Microorganisms and molecular identification

The most promising isolates (antibiotic resistant isolates) were subjected to phenotypic identification using cultural characteristics in a trail to be identified. Gram staining and analysing biochemistry. The region of 16S rRNA was amplified using the universal primers (F: AGAGTTTGATCMTGGCTCAG and R: TACGGYACCTTGTTACGACTT). PCR reaction, was performed for 4 min at 95°C followed by 40 cycles each of (40 sec at 94°C, 50 sec at 58°C and 50 sec at 72°C), followed by a supplementary 10min at 72°C. Sequences of the 16S rRNA genes were obtained from the NCBI database. Multiple alignments based on the most closely related sequences and similarity levels were carried out using the BLAST program1. A phylogenetic tree was reconstructed using the Mega 5 software.

2.4. Bacterial resistance determination using molecular techniques

Fresh bacterial cells were used to extract DNA using the GeneJET Genomic DNA Purification Kit. GEBRI kit removed plasmid from the bacteria's selected isolates.

2.4.1. Virulence gene detection using PCR

Twenty-one primers were designed; the genus used for screening of ESBL class A were TEM, CTX MUI, CTX BETA and BSHV (Table 1). ESBL class B carbapenemase encoding genus were CITM, VIM, IMP, KPC and NDM. ESBL class C encoding genus was FOX, ACCM. ESBL class D encoding genus was OXA-10, OXA-55, OXA58, OXA 60 and OXA 69. Screening for coding sequence of genes DHAM, and EBCM, MEC A, VAN A, and TOHO1 were evaluated. PCR reaction was achieved for 4 min at 95°C followed by 35 cycles each of: 40 sec at 94°C, 50 sec at (50-60) °C (according to primer) and 50 sec at 72°C, followed by a supplementary 10min at 72°C. After amplification by PCR, the products were checked in 2% agarose gel electrophoresis. Genetic miscellany was determined as the experimental number of differentiation. The primers used throughout the present work illustrated in (table 1).

3. Results

3.1. Sample collection and analysis of microbiology

To evaluate the incidence of isolates of bacterial ESBL and CR in certain dairy products at various locations in Alexandria, Egypt. A total of 100 samples were collected that were distributed as follows: Kareish cheese, Industrial white cheese, Old cheese, Romy cheese and milk (40, 28, 15, 10 and 7 %) respectively.

3.2. Assessment of isolated bacteria's resistance prevalence

All bacterial isolates (130) have been tested with different types of antibiotics for antibiotic resistance.Multiantibiotic resistance was shown by the biggest promising isolates (30). 7bacterial isolates were isolated from milk but show sensitive effect towards the antibiotic used for detection of MDR bacteria. All the selected MDR isolates were resistance to metronidazole (MTZ). Incidence of antibiotic sensitivity of multi-antibiotic resistance bacterial isolates present in table 2. Distribution of the MDR bacterial isolates were 11 isolates from kariesh cheese (out of 52 bacterial isolates), 5 isolates from

industrial white cheese (out of 19 bacterial isolates), 4 isolates from old cheese (out of 37 bacteria isolates) and 2 isolates from romy cheese (out of 13 bacteria isolates).

3.3. Identification of the bacterial isolates

To identify the bacterial selected with the most promising MDR (30 out of 130 isolates). The most commonly isolated pathogens were evaluated for phenotypic characterization and molecular identification using 16s rDNA. The amplified fragment sequences of 1500bp were easily identified. A dominant tool for identifying and classifying prokaryotes was the sequences of the different types of strains from gene bank. GenBank deposited the sequence of the most promising isolates and had the accession. From all the examined samples were *staphylococcussp*, *Bacillus sp*, *Salmonella sp, E.coli, Enterococcus sp, Pseudomonas sp, Shigella sp* and *Proteous sp.* with 43.3, 16.7, 13.3, 10, 6.7, 3.3, 3.3 and 3.3 % respectively. 23.1 % out of the total isolates (130) were pathogens.

3.4. Resistance determination using molecular technique

The variation between the isolated MDR bacteria were shown by plasmid extraction of the selected bacterial isolates. Results in figure (1) showed that isolates 2, 9, 16, 19, 20, 24, 25, 27, 29, 30, 39, 40 and 41 were highly antibiotic-resistant positive plasmid with high copy numbers.

3.4.1. Detection of virulence gene using PCR

3.4.1.1. Detection of β-lactamase gene class A, B,C and D in the selected bacterial isolate: Collected data of genes presence or absence for class A (TEM, CTX and BSHV), class B (VIM, IMP, KPC and NDM), class C (FOX) and class D (OXA-10, OXA-24 and OXA-58) were tested. The fragment size of amplified PCR product was calculated using software of Gel Documentation Analysis System (Alpha Imager TM 1220). Results in fig (2) showed the average percentage of positive genes in all the selected MDR (30isolates). Distribution of presence or absence of the 20 tested virulence genes among the MDR bacterial isolates (30 tested isolates) were illustrated in supplementary file (fig 1 - 19).

4. Discussion

Studies previously conducted in different countries revealed a wide *S. aureus* diversity in dairy products. The prevalence of *S. aureus* was 43.3% and it was differing than which carried out from Iran and Italy on different dairy products revealed a lower *S. aureus* percentage. Lower prevalence noted in studies of [20, 21, 22] who detected *S. aureus* with (10 %, 11.25 %, 5% and 9.1% respectively) of kariesh cheese samples. Greater incidence were informed by [23, 24, 25] as a result of which *S. aureus* was 72%, 50%, 70%, 93% and 68 % respectively of kariesh cheese samples.

Percentage of *Bacillus sp.* in our study was 16.7% and were isolated from industrial white cheese and kareish cheese where it was higher percentage in industrial white cheese. It was differed than the study by EFSA 2005 where the incidence of *B.cereus* was highest in karish cheese (25 to 80%) during all seasons followed by koshary (45 to 70%) and cornsnacks (20 to 50%).

Percentage of *Salmonella sp.* In our study was 13.3% that were isolated from industrial white cheese and kareish cheese (10% and 3.3 %). Relatively lower results were obtained by [26, 27, 28, 29, 30, 31] where salmonella species could not be identified in the surveyed white cheese samples. Ghada et al. (2004) identified two samples polluted with *Salmonella sp*.

In our study the *E.coli* percentage was 10% isolated from romy, kareish and industrial white cheese compared to other bacterial isolates. In study by Abdelrahman et al. (2019) found that the incidence of *E.coli* in Kariesh, and Domiati cheese were 37.1% and 2.8%, respectively. While, could not be detect in Tallaga cheese. Najand and Ghanbarpour (2006) who isolated *E.coli* with percentage of 98.70% from domestic soft cheese. Paneto et al (2007) isolated *E. coli* with percentage of 96% from raw milk cheese. Elsayed et al (2011) isolated *E.coli* with percentage of 40% from white soft cheese. Bonyadian et al. (2014) isolated *E. coli* with percentage of 48% from cheese samples. Ombarak et al. (2016) isolated *E.coli* from with percentage of 76.4%. Amer and Ewina (2003) and Eman (2015) where they isolated *E.coli* with percentage of 37.5%. While comparatively lower result were obtained by Brooks et al.(2012) as they found that 2 out 41 raw milk cheese samples were contaminated with *E.coli*.

A common principal of resistance markers in all varieties estimated and associated antimicrobials such as tetracyclines, B-lactams, sulfonamide and quinolones were detected [42].An aggregate number of extended-spectrum β-lactamases (ESBLs) have been

predictable in Enterobacteriaceae through the latest few years. SHV types of enzymes have been shown to carry SHV-1 gene within the chromosome [43]. NDM-1 producers were found resistant to imipenem, meropenem, ertapenem, gentamicin, amikacin, tobramycin, and ciprofloxacin, whereas, isolates were found susceptible to colistin [44]. The high prevalence of tetracycline- and penicillin-resistant (*S. aureus*, *Salmonella* and *E.coli*) observed in the current study, is in agreement with earlier findings. TEM are mostly preset by Gram-negative bacteria. Almost 90% of the resistance against ampicillin in Gram-negative bacteria are due to TEM encoded genes [46].

5. Conclusions

The results of this study indicate that cheese made from unpasteurized milk can pose a significant risk to consumers. This risk varies depending on the geographical location of the study area, the state of education of the population, the extent of attention to hygiene and the method of preparing and packaging dairy products.

Product manufacturing processes should be subject to health control-to-control pathogens. Dairy markets should be monitored for pathogens. Reducing the surface area exposed to air reduces harmful microbial growth in dairy products.

6. Declarations

Ethics approval and consent to participate: No need for it .

Consent for publication: All authors agree for publication.

Availability of data and material: All data available

Competing interests: No competing

Funding: This work has no fund

Authors' contributions:

WKA: Sample collection, screening of MDR bacteria and gene detection.

SMA: proposed the work, help student (Waleed) in the experimental part, funded fine chemical, wrote the manuscript and analyzed the experimental data

ZAO: supervised and revised the manuscript.

All authors agree for publication that work in BMC microbiology

Acknowledgements: Not Applicable

7. Abbreviations

Multidrug-resistant: (MDR), Antimicrobial resistance: (AMR), Metallobetalactamases: (MBLs), AmpC: Enzymes encoded by resident chromosomal genes (cAmpCs) are produced by some species, Extended-Spectrum Beta-Lactamase: (ESBL).

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Tables

Table 1: primers used in the work-study.

Gene	Reference
NDM: F: GGTTTGGCGATCTGGTTTTC, R: CGGAATGGCTCATCACGATC	[47]
KPC: F (CGTCTAGTTCTGCTGTCTTG), R: CTTGTCATCCTTGTTAGGCG **VIM**: F: GATGGTGTTTGGTCGCATA, R: CGAATGCGCAGCACCAG	[48]
TEM: F:AAAATTCTTGAAGACG, R: TTACCAATGCTTAATCA	[49]
DHAM: F: AACTTTCACAGGTGTGCTGGG, R: TCCGTACGCATACTGGCTTTGC	[50]
MECA: F:AAAATCGATGGTAAAGGTTGGC, R:AGTTCTGCAGTACCGGATTTTG	[51]
CTX beta: F: TTTGCGATGTGCAG(C/T)ACCAG, R:CGCGATATC(A/G)TTGGTGGTGCCATA	
OXA-58: F: AAGTATTGGGGCTTGTGCTG, R: CCCCTCTGCGCTCTACATAC	[52]
CITM: R: TTT CTC CTG AAC GTG GCT GGC, F: TGG CCA GAA CTG ACA GGC AAA	[53]
CTX: F: TTAGGAAATGTGCCGCTGTA R: CGATATCGTTGGTGGTACCAT,	[54]
CTX-MU1: F: ATGTGCAGYACCAGTAARGT, R: TGGGTRAARTARGTSACCAGA **ACCM:** F: AACAGCCTCAGCAGCCGGTTA R: TTCGCCGCAATCATCCCTAGC,	[55]
OXA 10: F: TCAACAAAT CGC CAGAGAAG, R: TCC CACACCAGAAAAACCAG	[56]
OXA-10: F: GCCATGAAAACATTTGCCGC, R: GCCACCAATGATGCCCTCAC	[57]
IMP: F: GTGGTTCTTGTAAATGCTGAGG , R: CCGCCTGCTCTAATGTAAGT	[58]

EBCM: F: TCGGTAAAGCCGATGTTGCGG, R:CTTCCACTGCGG CTG CCA GTT	[59]
OXA-69: F: CTAATAATTGATCTACTCAAG R:CCAGTGGATGGATGGATAGATTATC,	[60]
TOHO1: F: GCGACCTGGTTAACTACAATCC, R: CGGTAGTATTGCCCTTAAGCC	[61]
BSHV: F:ATGCGTTATATTCGCCTGT, R:TGCTTTGTTATTCGGGCCAA	[62]
VANA: F: GGGAAAACGACAA TTGC, R: GTACAATGCGGCCGTTA	[63]
FOX: F: CGAGCAGACSCTGTTCGAG R:TTGGCCAGCATGACGATG,	[64]

Table 2: Resistance prevalence of the bacterial isolates (30 isolate)

Antibiotics	Resistance % %	Antibiotics	Resistance % %
Colistin (CT)	66.7	Ceftazidime (CAZ)	90
Ampicilin +Sulbactam (A/S	90	Amikacin (A/K)	70
Penicilin(P)	83.3	Clindamycin (DA)	93.3
Chloramphenicol(C)	76.7	Rifampine(RA)	86.7
Ofloxacin (OFX)	83.3	Nitrofurantion (F)	76.7
Levofloxacin(LEV)	86.7	Cefoperazone (CFP)	86.7
Cefepime (CPM)	93.3	Azetreonum (ATM)	96.7
Meropeneme (MEM)	70.7	Tetracycline (TE)	83.3
Metronidazole (MTZ)	100	Streptomycine (S)	83.3
Doxycycline (DO)	83,3	Trimethoprim-sulfamethoxaz (STX)	80

Figures

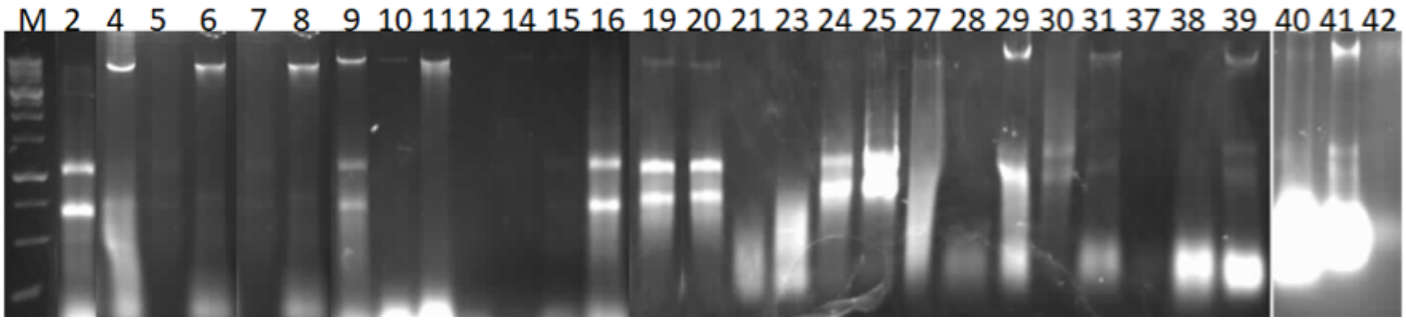

Figure 1

Agarose gel electrophoresis plasmid profiles of different bacterial isolates.

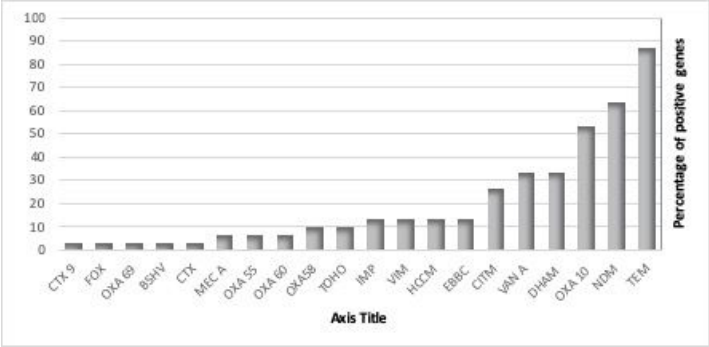

Figure 2

Average percentage of positive genes in all the selected MDR (30 isolates)