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Effects of a specific nutrient combination on ESBL resistance

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ABSTRACT

Background and aim: Extended-spectrum beta-lactamases are the main cause of resistance in Enterobacteriaceae to beta lactam antibiotics. The aim of this study was to evaluate the antimicrobial effect of EpiQuercican supplement, combined with different antimicrobial agents, on ESBL-producing isolates and determine the underlying molecular mechanism of resistance in these isolates.**Materials and methods:** Eleven ESBL producing Enterobacteriaceae isolates were collected from Saudi Arabia hospitals between 2016 and 2017 and disk diffusion test was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines to determine the susceptibility of the isolates to 5 different antibiotics in the presence of EpiQuercican supplement. Polymerase chain reaction was performed for detection of ESBL genes, and efflux pump inhibitor was used to study the mechanism of resistance in these isolates.**Results:** The best synergistic effect was obtained when the supplement was combined with carbapenems followed by 4th generation cephalosporins. Either no effect or antagonistic effect was seen with most of the isolates when the supplement was added to the 3rd generation of cephalosporins. Among the tested genes responsible for ESBL production in this study, our results indicated the predominance of TEM genes (73%) followed by CTX-M genes (9%). As for the mechanism of resistance in ESBL isolates, 4 isolates showed to use efflux pumps as their main mechanism of resistance.**Conclusion:** The EpiQuercican supplement showed some promising results, yet its antibacterial mechanism of action needs to be elucidated further.© 2018 Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The constant increase of antibiotic resistance in pathogenic bacteria is a topic of continuous review worldwide because of its severe consequences on the treatment of infectious diseases (Cosgrove, 2006; Tumbarello et al., 2010). Beta-lactams are the most common antimicrobial agents used to treat serious Gram-

negative infections; and as a result, resistance to beta-lactam antibiotics was developed through the production of beta lactamases (Bonnet, 2004; Bradford, 2001).

Beta-lactamase genes are widespread and subject to constant and dynamic mutations due to their prolonged exposure to a multitude of beta lactams (Bradford, 2001). This continuous exposure has led to the emergence of extended-spectrum beta-lactamases (ESBLs). ESBLs are plasmid mediated enzymes and confer resistance to bacteria through hydrolyzing third-generation cephalosporins and monobactams and rendering them ineffective (Bonnet, 2004; Paterson and Bonomo, 2005).

To this day, more than 350 different natural ESBL variants are identified and categorized into 9 separate evolutionary and structural families based on their amino acid sequences, which includes: TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA (Bajpai et al., 2017). Although some ESBL genes are mutant derivatives of beta-lactamases like *bla*_{TEM} and *bla*_{SHV} and have worldwide

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distribution, others are mobilized from environmental bacteria like *bla*_{CTX-M} and encode for the most widespread enzymes (Overdevest et al., 2011).

ESBL producing Enterobacteriaceae, mainly *Escherichia coli* and *Klebsiella pneumoniae* have increased dramatically during the past decades (Kim et al., 2002). This rise was a major cause of serious infections worldwide with high mortality and morbidity rates, which led to the increased reliance on last-resort antimicrobial drugs like carbapenems (Overdevest et al., 2011).

To overcome this problem, there has been growing interest to find new antimicrobial compounds from soil microorganisms, herbs, trace elements and minerals. Several studies conducted by researchers reported that by combining these substances with different antimicrobial agents, promising results were obtained (Fernández-Mazarrasa et al., 2009; Khan et al., 2005). For this purpose, Dr. Ruth's nutritive formula, EpiQuercican, was implicated in this study. Although the main purpose of this dietary formula is to support cell protection and normal cell functioning, yet most of its ingredients are implicated to support the function of the immune system and act as antimicrobials. Among the 10 micronutrients found in this formula, 7 ingredients have shown to have strong antibacterial properties including: Vitamin C, Green Tea extract, N-acetylcysteine, Quercetin, Selenium, Copper and Magnesium (Ibrahim et al., 2011; Kallio et al., 2012; Khiralla and El-Deeb, 2015; Robinson, 2011; Radji et al., 2013; Moon et al., 2016; Wang et al., 2017).

The aim of this study is to investigate the synergistic effect of EpiQuercican with a number of antimicrobial agents against ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, and correlate the underlying molecular mechanisms with the resistant phenotypic outcomes.

2. Materials and methods

2.1. Bacterial strains

Eleven Gram-negative ESBL-producing clinical bacterial isolates were collected from Saudi Arabia hospitals between 2016 and 2017. Using standard bacteriological methods, 7 of these isolates were identified to be *Klebsiella pneumoniae* and 4 were identified to be *Escherichia coli*. The isolates were stored in Brucella Broth with 10% glycerol at -80°C until use.

2.2. Antimicrobial agents and media supplement

Five antimicrobial agents were used in this study: Cefotaxime (30 μg), Ertapenem (10 μg), Imipenem (10 μg), Cefepime (30 μg) and Cefpodoxime (10 μg). The supplement used was a commercially available EpiQuercican™ (Dr. Rath Health Programs USA, BV 1260 Memorex Drive, Suite 200, Santa Clara, CA 95050). EpiQuercican contain in its formulation: Vitamin C, Calcium, Magnesium, Manganese, Selenium, Copper, Green tea extract, L-lysine, L-proline, L-arginine, and Quercican.

2.3. Antimicrobial susceptibility testing

The susceptibility of 11 ESBL producing clinical isolates to the 5 antimicrobial agents was evaluated by performing Kirby-Bauer disk diffusion test in the absence or presence of the supplement following CLSI protocol. For this purpose, the plates contained either Mueller Hinton agar alone or Mueller Hinton agar supplemented with EpiQuercican at a concentration of 200 $\mu\text{g}/\text{plate}/25\text{ ml}$. Each plate was then inoculated uniformly with 0.5 McFarland of bacterial inoculum by the full plate streaking method. Antibiotic discs were then placed on the agar plates and

the plates were incubated at 37°C for 24 h. After 24 h of incubation, the plates were observed for zone of inhibition around the antibiotic discs. The zone of inhibition for different antibiotics was measured and the isolates were classified as either susceptible or resistant according to the interpretative standards recommended by the CLSI.

2.4. Plasmid extraction

Plasmid DNA was extracted using QIAGEN Plasmid Mini Kit (QIAGEN GmbH, D-40724 Hilden) and the procedure was carried out according to the manufacturers' protocol. The DNA was stored at -20°C until use.

2.5. Polymerase chain reaction assay

ESBL-encoding genes, *bla*_{TEM-1} and *bla*_{CTX-M-15}, among the 11 ESBL-producing isolates were analyzed by PCR amplification using the following specific primers: The gene CTXM-15 was amplified using forward primer 5'GGTAAAAAATCACTGCGTC-3' and reverse primer 5'-TTACAAACCGTCGGTGACGA-3'; and the gene TEM-1 was amplified using the forward primer 5'ATGAGTATTCAACATTTCG-3' and reverse primer 5'-CCAATGCTTAATCAGTGAGG-3'. Amplification was achieved using the PCR Sprint Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions were optimized in house previously, with an annealing temperature of 50°C . PCR amplicons were electrophoresed on 1.5% agarose gel using SeaKem® LE Agarose (FMC BioProduct, Rockland, ME, USA). Ten microliters of each sample was mixed with two microliters of the loading dye (Fermentas Life Sciences, Burlington, Ontario, Canada) before being loaded into their respective wells. A 100 base pair ladder (Fermentas Life Sciences, Burlington, Ontario, Canada) was run in parallel to the samples and served as a size marker. Amplicons were then observed using ULTRA LUM, Dual Light Transilluminator (Claremont, CA), and photographed using a digital camera (Olympus). IMP 21, 28 & 53 are used as positive controls for TEM-1 and IMP 44 & 2 are used as negative control.

2.6. Efflux pump inhibitor tests

To study the effect of efflux pump inhibitor on the susceptibility of ESBL-producing *Escherichia coli* and *K. pneumoniae* isolates to cefotaxime, disk diffusion test was performed according to CLSI guidelines. The inhibitor used in our study was the phenylalanine arginine- β -naphthylamide dihydrochloride (PA β N) (Sigma-Aldrich Co., St Louis, MO). Mueller Hinton agar plates containing either 100 $\mu\text{g}/\text{mL}$ of (PA β N) inhibitor alone or along with the supplement were inoculated uniformly with 0.5 McFarland of bacterial inoculum by full plate streaking method. Cefotaxime antibiotic disc was then placed on the Mueller Hinton agar plates and the plates were incubated at 37°C for 24 h. After 24 h of incubation, the plates were observed for zone of inhibition around the antibiotic disc and were measured. The isolates were classified as either susceptible or resistant according to the interpretative standards recommended by the CLSI.

3. Results

3.1. Antimicrobial susceptibility testing

The results of the antimicrobial susceptibility testing of 11 ESBL producing *K. pneumoniae* and *E. coli* clinical isolates to 5 antibiotics tested in the presence of supplement revealed that the best synergist effect was seen when the supplement was combined with carbapenems. In summary, all of the tested isolates showed an

increase in their diameter of zone of inhibition by an average of 0.51 mm when tested for imipenem susceptibility. As for ertapenem, 10 out of 11 tested isolates showed an increase in their diameter by an average of 0.79 mm while one isolate did not show any change in its zone of inhibition diameter. As for cephalosporins, better results were obtained with 4th generation cephalosporins rather than 3rd generation cephalosporins. When tested for cefotaxime, 4 isolates showed an average increase of 0.33 mm in their diameter of zone of inhibition, 6 isolates did not show any change in their diameter and 1 isolate showed a 0.5 mm decrease in its diameter. Finally, when the 11 isolates were tested for cefpodoxime in the presence of the supplement, 3 isolates showed an average increase of 0.8 mm in their diameter, 2 isolates did not show any change in their diameter and 6 isolates showed an average decrease of 0.75 mm in their diameter of zone of inhibition. None of the resistant isolates reverted back to susceptible except for one *K. pneumoniae* isolate which showed intermediate resistant when the supplement was added to cefpodoxime (Table 1). This experiment was repeated twice, and consistent results were obtained.

3.2. ESBL-encoding genes detection

The amplification of ESBL encoding genes revealed that 8 out of the 11 tested isolates harbored *bla*_{TEM-1} and only one isolate (0520E) harbored the *bla*_{CTXM-15}. Three isolates (0507K, 0508K, and 0523E) did not harbor any of the tested genes. One isolate (0520E) showed to have both *bla*_{TEM-1} and *bla*_{CTXM-15} (Fig. 1A, B and Table 2). This experiment was repeated twice, and consistent results were obtained.

3.3. Efflux pump inhibitor

Out of the 11 ESBL-producing isolates tested for cefotaxime in the presence of PAβN inhibitor, 4 isolates (0523E, 0517E, 0520E, and 0511E) showed an increase in their zone of inhibition diameters (≥ 26 cm) when compared to the plates containing the antibiotic alone or to the plates containing the supplement. The supplement didn't have any effect on the activity of the inhibitor in this case, because the plates containing the PAβN inhibitor alone also showed the same outcome. Moreover, no difference in the

diameters of the zone of inhibition of 6 isolates was seen. Finally, a decrease was observed in the diameter of the zone of inhibition of one isolate (0511 K) by 0.1 mm when the inhibitor was added both in the presence and absence of the supplement (Table 3). This experiment was repeated twice, and consistent results were obtained.

4. Discussion

The rapid increase of ESBL producing Enterobacteriaceae is a threat to the public health, because of the poor clinical outcomes for patients infected with ESBL producing bacteria. ESBLs are plasmid encoded, and their easy transfer to different hosts has facilitated to the pathogens the ability to confer resistance against novel antibacterial agents through the production of new beta lactamases (Cosgrove, 2006; Bradford, 2001; Tumbarello et al., 2010). Among the tested genes responsible for ESBL production in this study, our results indicated the predominance of TEM genes (73%) followed by CTX-M genes (9%). These outcomes are in accordance with Yazdi et al. (2012) and Bajpai et al. (2017) who also showed the prevalence of TEM genes over the CTX-M genes. However, this data varies largely among published articles. Several papers showed that the frequency of CTX-M gene exceeded that of the TEM (Ahmed et al., 2013; Eftekhar et al., 2012; Shahid et al., 2011). This led us to the conclusion that the type and the occurrence of ESBL genes can differ from one geographical region to another. In fact, a study from China showed the predominance of TEM gene in ESBL isolates, yet another study reported CTX-M to be the predominant gene in New York, Spain, and United Kingdom (Ahmed et al., 2013; Eftekhar et al., 2012; Shahid et al., 2011). Moreover; in our study, 3 isolates (27%) lacked the tested TEM and/or CTX-M genes yet remained phenotypically ESBL positive. This can be explained by the fact that these isolates most probably harbored other ESBL encoding genes.

A common mechanism responsible for multidrug resistance among pathogenic bacteria to antibiotics is the efflux pumps. These active drug transporters render the bacteria resistant through several mechanisms including cross-resistance to chemically distinct classes of molecules and low intrinsic susceptibility (Mahamoud et al., 2007; Laudy et al., 2016). By targeting these efflux mechanisms, the intracellular antibiotics concentration can

Table 1
Disk diffusion susceptibility testing of ESBL-producing isolates treated with cephalosporins and carbapenems in the presence of EpiQuercian supplement (k = *Klebsiella pneumoniae*, E = *Escherichia coli*, S = supplement).

Isolate type	Isolate number	Cefotaxime (CTX)	Ertapenem (ETP)	Imipenem (IPM)	Cefepime (FEP)	Cefpodoxime (CPD)
ESBL	0508 K	1.2/R	3/S	2.8/S	2/S	0/R
ESBL	0508 K + S	2.0/R	3.6/S	3/S	2/S	1.1/R
ESBL	0523 E	1.7/R	3.1/S	3.1/S	1.9/S	1.2/R
ESBL	0523 E + S	1.8/R	3.9/S	4/S	1.9/S	1.4/R
ESBL	0517 E	1.8/R	3.3/S	3.3/S	2.3/S	1/R
ESBL	0517 E + S	1.8/R	3.8/S	3.6/S	2.3/S	1/R
ESBL	0519 K	1.6/R	3.2/S	3.1/S	2.2/S	1.2/R
ESBL	0519 K + S	1.6/R	3.8/S	3.5/S	2.1/S	0/R
ESBL	0520 E	1.2/R	3/S	3/S	1.7/I	0/R
ESBL	0520 E + S	1.2/R	3/S	3.1/S	1.7I	0/R
ESBL	0507 K	1.7/R	3.2/S	3.2/S	2.2/S	1.0/R
ESBL	0507 K + S	1.7/R	4.0/S	3.6/S	2.3/S	0/R
ESBL	0512 K	1.6/R	3.2/S	3.2/S	2.3/S	1.1/R
ESBL	0512 K + S	1.6/R	4.1/S	3.5/S	2.5/S	0/R
ESBL	0515 K	2.0/R	3.2/S	3.2/S	2.3/S	0.9/R
ESBL	0515 K + S	1.5/R	4.0/S	4.0/S	2.4/S	0/R
ESBL	0513 K	1.6/R	3.1/S	3.0/S	2.3/S	0.9/R
ESBL	0513 K + S	1.6/R	4.0/S	4.0/S	2.4/S	0/R
ESBL	0518 E	1.6/R	3.0/S	3.0/S	2.1/S	0.9/R
ESBL	0518 E + S	1.8/R	4.2/S	3.6/S	2.4/S	0/R
ESBL	0511 K	1.7/R	3.1/S	3.0/S	2.2/S	0.9/R
ESBL	0511 K + S	1.9/R	3.9/S	3.6/S	2.5/S	2.0/I

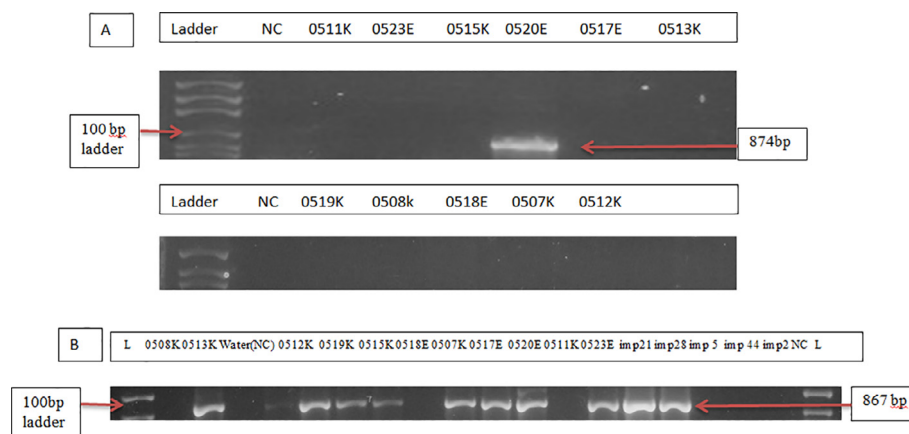


Fig. 1. Prevalence of ESBL genes in Enterobacteriaceae. (A) PCR gel result of CTX-M gene. (B) PCR gel result of TEM gene.

Table 2

Summary of molecular analysis of *Escherichia coli* and *Klebsiella pneumonia* harboring ESBL encoding genes.

Isolate Number	CTXM-15	TEM-1
0507 K	Negative	Negative
0508 K	Negative	Negative
0511 K	Negative	Positive
0512 K	Negative	Positive
0513 K	Negative	Positive
0515 K	Negative	Positive
0517E	Negative	Positive
0518E	Negative	Positive
0519 K	Negative	Positive
0520E	Positive	Positive
0523E	Negative	Negative

Table 3

Disk diffusion result of ESBL-producing isolates treated with Cefotaxime (CTX) in the presence of EpiQuercican supplement and the PaβN inhibitor.

Isolate type	Isolate number	Cefotaxime (CTX) –PaβN	Cefotaxime (CTX) +PaβN
ESBL	0508 K	1.2/R	1.2/R
ESBL	0508 K + S	2.0/R	2.0/R
ESBL	0523 E	1.7/R	≥26
ESBL	0523 E + S	1.8/R	≥26
ESBL	0517 E	1.8/R	≥26
ESBL	0517 E + S	1.8/R	≥26
ESBL	0519 K	1.6/R	1.6/R
ESBL	0519 K + S	1.6/R	1.6/R
ESBL	0520 E	1.2/R	≥26
ESBL	0520 E + S	1.2/R	≥26
ESBL	0507 K	1.7/R	1.7/R
ESBL	0507 K + S	1.7/R	1.7/R
ESBL	0512 K	1.6/R	1.6/R
ESBL	0512 K + S	1.6/R	1.6/R
ESBL	0515 K	2.0/R	2.0/R
ESBL	0515 K + S	1.5/R	1.5/R
ESBL	0513 K	1.6/R	1.6/R
ESBL	0513 K + S	1.6/R	1.6/R
ESBL	0518 E	1.6/R	≥26
ESBL	0518 E + S	1.8/R	≥26
ESBL	0511 K	1.7/R	1.7/R
ESBL	0511 K + S	1.9/R	1.7/R

be increased and the resistant pathogens can be restored back to their susceptible phenotype (Mahamoud et al., 2007; Laudy et al., 2016). For this purpose, we performed susceptibility testing in the presence and absence of efflux pump inhibitor PaβN combined with the supplement. Among the 11 tested isolates, only 4 isolates showed significant increase in their diameter of zone of inhibition.

This implies that the main mechanism of resistance among these 4 isolates is through the use of efflux pumps. It is worth noting that the supplement didn't aid the inhibitor in converting these resistant isolates into susceptible. It is possible that the antibacterial ingredients found in the supplement don't target the bacterial drug transport system but exert their effect using different mechanisms which needs to be explored further. While for the remaining 7 isolates, it appears that the mechanism of resistance in these isolates is not related to the efflux pump activity.

As for the antimicrobial susceptibility testing with EpiQuercican, synergistic effect was obtained when the supplement was combined with carbapenems and antagonistic effect was seen when the supplement was combined with cephalosporins, yet several variations within each group were observed. In case of the 3rd generation cephalosporin, cefpodoxime, either no effect or antagonistic effect was seen with most of the isolates; however, two isolates showed an increase in their diameter when the supplement was added, and one isolate changed from being resistant to having intermediate susceptibility to cefpodoxime. A similar scenario occurred with ertapenem, whereby most of the isolates showed increase in their diameter of zone of inhibition when the supplement was added yet 1 isolate did not show any change. These deviations led us to the conclusion that the effect seen, regardless synergistic or antagonistic, is independent of the drug in use and has to do with the tested isolate itself. A possible explanation might be that the ingredients found in the supplement can interfere with ESBL genes and inhibit their function. In fact, a recent study published by Djoko et al. (2018) showed that copper has the ability to inhibit the activity of NDM-1 in carbapenem resistant Enterobacteriaceae and increase their susceptibility to carbapenems.

5. Conclusion

It appears that EpiQuercican supplement possesses some bactericidal properties, yet the exact mechanism of action of this supplement needs to be investigated further. Moreover, improved results can be obtained by increasing the population number and broadening the number of ESBL genes screened.

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Authors contributions

Steve Harakeh and Esam Azhar: Designed this study, confirmed isolates using molecular techniques, revised and approved the final version of this paper.

Saad Almasaudi: Collected clinic samples for the project, cultured and isolated suspected colonies for further identification using basic microbiological techniques.

Kohar Berge Kissoyan, Sukayna Fadlallah and Arax Tanelian: Performed phenotypic and molecular testing of the samples, data analysis and interpretation, assisted in drafting the manuscript and approved the final version of this paper.

Ghassan Matar: Contributed to the conception and design of this study, drafted and had critical revision of the manuscript and approved this final version.

Conflict of interest

The authors declare that they have no conflict of interests.

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