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Study of The Anti-bacterial Efficacy of Probiotics Culture Extracts on Extended Spectrum Beta Lactamase-Producing Multi-Drug Resistant Isolates, Bearing BLA-TEM-1 Gene, in Patients with Wound Infections in Hospitals

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ABSTRACT

Background: Infections caused by Extended Spectrum Beta Lactamase- producing wound pathogens are difficult to be eradicated worldwide. The prevalence rates were reported as worldwide rising, Therefore, Imperative work is needed to detect safe, non-toxic and new-effective Therapy.

The Aim of The Work: To detect the first gene was isolated from female patient called temonira-1 gene, bearing Extended Spectrum beta Lactamase-producing strains among multi-drug resistant bacteria isolated from patients with wound infections. And to evaluate the antibacterial effects of PROBIOTICS' culture extracts on these isolates.

Patients and Methods: Overall, 320 patients selected from those with wound infections seen in Egypt, The Wound swab samples were collected, transported in brain heart infusion broth, isolated in pure culture and bacterial species were identified by the conventional microbiological methods. Antimicrobial susceptibility testing were done, followed by Phenotypic Detection of Extended Spectrum Beta Lactamase production using the double disk synergy test, PCR technique was done to detect isolates bearing the gene. The detected isolates had tested by the PROBIOTICS' culture extracts, using the well-agar diffusion method and the reducing colony count activity technique.

Results: *[48.54%]* of the total multi-drug resistant pathogens identified were positive for ESβL by double disk synergy test. Among 100 ESβL-producers screened, 32 isolates were detected bearing $_{TEM-1}$ gene that coding for these enzymes by using PCR technique. All of the studied ESβL producers, bearing $_{TEM-1}$ gene were susceptible to PROBIOTICS' CULTURE EXTRACTS with inhibition zones diameter average in-between 12 and 21 mm., in addition, [65.6% of strains] showing reducing colony count activity by 100 % [zero CFU].

Conclusion: PROBIOTICS' CULTURE EXTRACTS revealed excellent activity against ESβL-producing microorganisms that bearing $_{TEM-1}$ gene. So, these extracts may be considered safe therapeutic promising options to treat such infections.

Keywords: Wound Pathogens; Extended SPECTRUM Beta Lactamases; Probiotics' Culture Extracts.

INTRODUCTION

Extended Spectrum βeta Lactamase are enzymes have the ability to develop microorganism resistance to penicillins, all generations of cephalosporins, and monobactams as they can hydrolyze those antimicrobial drugs. In addition, they do not affect carbapenems [1].

The TEM-1 is the most ordinarily coding gene in *Enterobacteriaceae* species as *E. coli* and *K. pneumoniae*. In addition to non-*Enterobacteriaceae* species as *Pseudomonas aeruginosa* [*P. aeruginosa*] [2].

The multi-drug resistances have a great interest and wish to be explained continuously. The transfer of drug resistance genes among wound pathogens making issues in therapeutic outcome worldwide [3]. So, antimicrobial therapy of wound infections caused by multi-drug resistant wound pathogens is hugely difficult. The choices of antimicrobial drugs are terribly restricted, and need urgent studies to search for new effective therapy [4].

Carbapenems are thought to be unique antimicrobials have the ability to eradicate ESβL-producers. However, carbapenem resistance has also additionally been more and more reportable in several countries later on [5].

On the other hand, attention is focusing on using PROBIOTICS' supplements containing benign microbes. PROBIOTICS are culture extracts of viable microbes such as *lactobacillus* that give profit by acting through certain mechanisms. They have an antibacterial efficacy, prevent the adhesion of bacteria to the epithelium, consume the nutrients necessary for bacterial survival and produce antitoxic substances [bacteriocin like] [6].

THE AIM OF THE WORK

The current work designed to detect the first gene was isolated from female patient called temonira [TEM-1] gene, bearing Extended Spectrum beta Lactamase-producing strains among multi-drug resistant- facultative anaerobic bacteria isolated from patients with wound infections. And to evaluate the antibacterial effects of PROBIOTICS' culture extracts on these isolates.

PATIENTS AND METHODS

The cross sectional study was conducted on 320 patients selected from those with wound infections seen in surgical department "out and inpatients" at Al-Azhar University Hospital in New Damietta, over the period between May 2020 and January 2022.

Inclusion Criteria:

- All ages and genders of patients suffered from wound infections had included in the study.
- Out of 320 patients, 100 ESβL-producing facultative anaerobic bacteria selected from the strains that confirmed with multi-resistance pattern were included in the study.
- Out of 100 ESβL-producing isolates, the isolates that bearing TEM-1 gene had subjected for PROBIOTICS' study.

Exclusion Criteria:

- All samples showed no bacterial growth was excluded from the study.

Wound Sample Collection:

Wound swab samples were collected under complete aseptic condition.

Sample Processing, for each specimen the following was done:

1- Transportation to the laboratory department of medical microbiology and immunology at the faculty of medicine, Damietta. The swabs were placed in Brain Heart Infusion broth and transported within 20 minutes.

2- Isolation in pure cultures, Samples had cultured on MacConkey and Nutrient agar media.

3- Complete identification of all isolates by the conventional microbiological methods as described by Collee *et al.* [7].

Antimicrobial susceptibility testing techniques:

It was performed for the isolates to confirm the multidrug resistance [non susceptibility to at least one drug in three or more antimicrobial categories]. This was carried out by using Kirby-Bauer susceptibility testing technique; the following commercially available disks were been tested: *[Amoxicillin, Gentamicin, Ciprofloxacin, Ceftazidime and Imipenem*] as described by *Bauer et al.* [8].

Detection of ESβL by the double disk Synergy method:

All multi-drug resistant isolates were being tested for the production of ESβL enzymes by using Double Disk Synergy Test [DDST]. Ceftazidime [30μg] disks were being applied on the inoculated plate, where a Ceftazidime disk were be placed 1.5 cm away from an Amoxicillin-Clavulanic acid disk [10μg]. An enhanced inhibition zone between the two disks [often coined or the keyhole phenomenon] is indicated for the ESβL production [9].

Detection of βLA*TEM-1* gene bearing isolates among ESβL-producing strains using PCR [Figure 1]**:**

All multi-drug resistant ESβL-producers were being tested for the presence of **βLA *TEM-1*** gene, using primer Sequence as described in table 1 [3,10].

PCR products and 100 bp~1000 bp DNA ladder Marker were included in gel analysis [10,11].

"Table 1": "Primer details of βLA *TEM-1* gene."

Primer	Primer sequence	Product size
βLA *TEM-1*	TGGATCTCAACAGCGGTA TTTATCCGCCTCCATCCA	893 bp

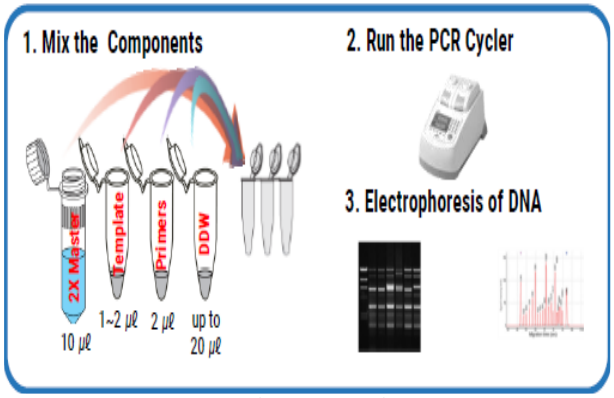

Figure [1]: Quick Guide for detection of **βLA** *TEM-1* gene by PCR.

Antibacterial activity of PROBIOTICS' metabolites:

The used PROBIOTICS' bacteria *[A mixture from* 10^6 *Lactobacillus delbrueckii* and 10^6 *Lactobacillus Fermentum]* were kindly provided from scientific research centre. Cultivation of PROBIOTICS'-bacteria in fluid media with special growth factors [MRS broth media/Oxoid, England]. It contains sodium acetate, in additional to the nutritional growth factors, which suppresses the growth of many competing bacteria as described by Gomez *et al.* [12].

For the filtration of cell-free supernatants of *lactobacilli*, about 16 ml. of MRS-broth media was inoculated with a mixture of the used *lactobacilli strains* and incubated at 30-37°C for 18-24 hrs. Then, the samples were centrifuged well for about 16 min., then, a [0.22 um]

Millipore-filter was used for filtration of the supernatants and used freshly. The Antibacterial activity of PROBIOTICS' extracts was determined by the following:

1- ESβL-producing strains, bearing **βLA*TEM-1*** gene were screened for their susceptibility to PROBIOTICS' extracts using the **agar well diffusion method** to determine the inhibition zone diameter [13]. Each isolate was swabbed and sub cultured overnight on Muller Hinton agar plates. Then, wells of 5 mm diameter were obtained. PROBIOTICS' liquid culture filtrates [100ul] were pipetted into the wells. The plates were then incubated at 37°C for at least 24 h.; the diameters of inhibition zone around the well were measured [14,15].

2- **Bacterial colony counts** were done twice as described by Sondi and Salopek [16], using a calibrated loop and Muller Hinton agar plates, the first time using PROBIOTICS' filtrates-free agar plates as control plates and the second time using PROBIOTICS' filtrates-included agar plates as tested plates [1.5 ml PROBIOTICS' filtrate were added to the agar during the original preparation and distributed uniformly by spread pouring method and swirling], ESβL-producing strains, bearing βLA*TEM-1* gene samples were be gently mixed by swirling and a calibrated loop were be selected [which delivers 0.01ml of wound samples], a loop-full of samples were inoculated on the selected media. The inoculated bacteria were distributed uniformly on agar surface plates, and then incubated at 37±1^{oC} for bacterial growth and the numbers of colonies were counted after 24 hrs.' As CFU = colony forming units for both control and tested plates to be compared together.

Ethical approval:

The protocol was approved by the institutional research and ethics committee [Faculty of medicine, Al-Azhar University, Egypt_0000013, May 2020]. Informed consent was obtained from the patients before including them in the study.

RESULTS

The following results were obtained:

Of the total patients, 58.75% were male and 41.25% were female. About 22.8% of the total patients were in the age in between 0 -11 years and 50 -60 years. Of the total patients, 128 were associated with DM, 140 were associated with previous surgical operations and 269 were on previous antibiotics medication. Of the total patients, 93 were associated with Abscess, 121 were associated with Trauma and 96 were associated with Surgery. 302 growing microorganisms had been isolated, and identified as shown in [Table 2].

Proteus and *E. coli* were the most wound pathogens isolated [Table 2]. Most isolates were multi-resistant to the antimicrobials. Amoxicillin shows the highest resistance rate [Table 3]. 206 of the isolates were resistant to > or = [3] antibiotics of different categories, [AMX: Amoxicillin] - [CIP: Ciprofloxacin] - [GN: Gentamycin] - [CAZ: Ceftazidime] as shown in [Table 4].

Out of 302 wound pathogens, 206 identified multi-drug resistant isolates had tested for the production of ESβL by double disk diffusion method. [48.54%] of the multi-drug resistant wound pathogens identified showed positive results. Of 54 E. coli, 33 were positive. Of 25 *Staphylococci*, 0 were ESβL positive. Of 52 *Klebsiella*, 36 were positive. 37 of the isolated *Proteus* were ESβL positive as shown in table [5].

Out of 100 isolates identified as ESβL producers, 32 bacterial isolates were been bearing TEM-1 gene as shown in [Table 6]. All ESβL-Bacterial Strains bearing TEM-1 gene were susceptible to PROBIOTICS'

CULTURE EXTRACTS. Pseudomonas shows the least susceptibility [12 mm of inhibition zone], the greater activity against *E. coli* was [21 mm of inhibition zone] in one strain. The greater activity for *proteus* and *Klebsiella* was [20 mm of inhibition zone] for two and one strains, respectively, as shown in [table 7].

All ESβL- producing strains bearing TEM-1 gene were susceptible to PROBIOTICS' CULTURE EXTRACTS. *Pseudomonas* shows the least susceptibility [50% reducing colony count], the greater activity against *E. Coli* was [100% reducing colony count] in 12 strains that show zones of inhibition average in-between 17 and 21 mm. While the greater activity for the *proteus* was [100% reducing colony count] for 3 strains that show zones of inhibition average in-between 18 and 20 mm. And the greater activity for the *Klebsiella* was [100% reducing colony count] for 6 strains that show zones of inhibition average in-between 17 and 20 mm as shown in [Table 8].

Table [2]: Identification of the isolates.

Name of microorganisms	*Number*	*Percentage %*
Proteus	80	26.49%
E.coli	70	23.1%
Klebsiella	62	20.52%
Staph. Aureus	33	10.92%
Staph. Epidermidis	21	6.95%
Pseudomonas	8	2.64%
Mixed	28	9.27%
Total	*302*	*100%*

Table [3]: Antimicrobial susceptibility of isolated wound-pathogens.

Name of Antibiotics	*Disk code*	*Potency*	*Percentage of Antibiotic resistance*
Amoxicillin	AMX	30ug	71.8% [217]
Ciprofloxacin	CIP	30ug	63.9% [193]
Gentamicin	GN	10ug	68.54% [207]
Ceftazidime	CAZ	30ug	58.27% [176]
Imipenem	IPM	10ug	0 % [0]

Table [4]: Distribution of microorganisms according to the number and patterns of antimicrobial resistances.

Patterns and Number of antibiotics resistances	*Number of Isolates resistances and Percentages*
No Antibiotics resistances **→[54]**	[96] →[31.7%]
Resistance to [1] Antibiotic: **[AMX]→[15]** **[GN]→[11]**	
Resistance to [2] Antibiotics: **[AMX, GN]→ [13]** **[GN, CIP] →[2]** **[CAZ, AMX]→[1]**	
Resistance to [3] Antibiotics: **[AMX, CIP, GN]→ [31]** **[AMX, GN, CAZ] →[15]** **[AMX, CIP, CAZ]→ [25]** **[GN, CAZ, CIP]→ [18]**	89→[29.4%]
Resistance to [4] Antibiotics: **[AMX, CIP, GN, CAZ] →[117]**	117→[38.7%]
Total	*302*

Table [5]: Detection of ESβL-positive bacterial wound pathogens by double disc synergy tests.

Name of organisms	*Number of Multi-drug resistant isolates*	*ESβL [Positive]*	*ESβL [Negative]*
E. coli	54	33 [47.1%]	21
Staphylococcus Aureus	25	0 [0%]	25
Klebsiella	52	36 [58 %]	16
Proteus	67	30 [37.5%]	37
Pseudomonas	8	1 [12.5%]	7
Total	*206*	*100[48.54%]*	*106*

Table [6]: ESβL-Positive bacterial pathogens bearing TEM-1 gene.

Name of organisms	*[Positive] ESβL by double disk-diffusion method*	*ESβL-producing strains, bearing TEM-1 gene*
E. coli	33	20 [60.6 %]
Klebsiella	36	8 [22.2%]
Proteus	30	3 [10%]
Pseudomonas	1	1 [100 %]
Total	*100*	*32 [32%]*

Table [7]: Inhibition Zone Diameters *[mm]*, Induced by CFS-PROBIOTICS' filtrates on ESβL-producing strains bearing TEM-1 gene.

ESβL-Bacterial Strains bearing TEM-1 gene	*E. Coli*	*Pseudomonas*	*Proteus*	*Klebsiella*
	Inhibition zone diameter in *[mm]*			
Strain 01	20	12	20	18
Strain 02	19		20	19
Strain 03	18		18	18
Strain 04	21			18
Strain 05	15			17
Strain 06	15			16
Strain 07	14			20
Strain 08	17			15
Strain 09	16			
Strain 10	15			
Strain 11	18			
Strain 12	16			
Strain 13	14			
Strain 14	19			
Strain 15	20			
Strain 16	17			
Strain 17	17			
Strain 18	16			
Strain 19	18			
Strain 20	17			

Table [8]: Bacterial colony counts of ESβL-producing strains that bearing TEM-1 gene using PROBIOTICS' filtrates-free agar plates as a control plates and PROBIOTICS' filtrates-included agar plates as test plates.

ESβL -Bacterial Strains bearing TEM-1 gene	*E.Coli: Colony counts [CFU/ML]*		*Pseudomonas: Colony counts [CFU/ML]*		*Proteus: Colony counts [CFU/ML]*		*Klebsiella: Colony counts [CFU/ML]*	
	Control Plates	Test Plates	Control Plates	Test plates	Control Plates	Test plates	Control Plates	Test plates
Strain 01	10^3	Zero	10^8	10^4	10^3	Zero	10^3	zero
Strain 02	10^3	Zero			10^4	Zero	10^2	zero
Strain 03	10^2	Zero			10^3	Zero	10^3	zero
Strain 04	10^4	Zero					10^2	zero
Strain 05	10^5	10^3					10^2	zero
Strain 06	10^4	10^2					10^4	10^2
Strain 07	10^5	10^4					10^3	zero
Strain 08	10^3	Zero					10^3	10^2
Strain 09	10^3	10^2						
Strain 10	10^4	10^3						
Strain 11	10^3	Zero						
Strain 12	10^5	10^3						
Strain 13	10^6	10^4						
Strain 14	10^4	Zero						
Strain 15	10^4	Zero						
Strain 16	10^3	Zero						
Strain 17	10^3	Zero						
Strain 18	10^3	10^2						
Strain 19	10^2	Zero						
Strain 20	10^2	Zero						

DISCUSSION

The study was conducted on 320 patients with wound infections. The isolates were 70 *E. coli*, 80 *proteus*, 62 *Klebsiella*, 33 *staph. Aureus* and others as identified using the conventional microbiological methods. Previous studies also showed *proteus* to be the most prevalent pathogens isolated from Wound samples [17]. In this study, the emergence of ESβL-producers among multi-drug resistant wound pathogens using double disk synergy method was *48.54%*. Of the ESβL-positive isolates, *Klebsiella* was the prevalent organism and this result was different from Khan *et al.* [18] who showed that *E. coli* was the most prevalent. In this study, the percentage of ESβL-producers was higher among *Klebsiella* [58 %] than *E. coli* [47.1%]. Similar findings were found by Fam and EL-Damarawy [19] that showed higher rates of ESβL-producing isolates among *Klebsiella* than *E. coli*. While Tsering *et al*. [20], Fennel *et al*. [21] and Ines *et al*. [22], accounted *E. coli* as 41.9%, 90.9%, and 72% and against *Klebsiella*; 24.6%, 5.6% and 18%, respectively. Other studies as described by Shahid *et al.* [23] showed higher rates of ESβL-producing *Klebsiella* than ESβL-producing *E. Coli.* Therefore, the emergence rates of ESβLs- producing *Klebsiella and E. coli* has rising globally [23,24]. Therefore, there is a need for detection of the most prevalent gene coding for the emergence of ESβL production [24,25].

Out of the studied 100-isolates identified as ESβL producers, [32%] bacterial isolates were been bearing TEM-1 gene detected by PCR technique. *E. coli* was the most prevalent isolates bearing this gene [60.6 %] followed by *Klebsiella.* Other studies reported that the prevalence of βLA*TEM-1* gene among *E. coli* was [61.1%] and [60.8%] as described by Ahmed *et al.* [26] and Al-Agamy *et al.* [27] respectively. Another study as described by Al-Mayahi *et al.* [28] who reported that, among the total isolates of *E. coli* and *K. Pneumonia*, βLA TEM genes were the most encountered ESβL-coding genes. It was showed in 81.6% among the ESβL-producing isolates, including [83.3%] of the ESβL-producing *K. Pneumonia* and [79.4%] of the ESβL- producing *E. coli.* In this study, all isolates were tested by different antimicrobial drugs. These drugs included aminoglycosides [Gentamycin], quinolones [Ciprofloxacin], Cephalosporins [Cefta-zidime] and others. Highest resistance rate was against amoxicillin [71.8%] and Gentamicin [68.54%].

The resistance rates to ciprofloxacin were 63.9%, this was agreed with a study carried out in Spain by Mata *et al.* [29] that showed [51.3%] resistance to Ciprofloxacin. Most of the isolates were resistant to more than three antibiotics. This may be explained by the fact that misuse of antimicrobials for treatment of common infections is hugely wide [30]. Thus, antimicrobial-policies are mandatory for proper hospital infection control [31]. All isolates were sensitive from the start to carbapenem by 100%. This was relatively in agreement with Kiffer *et al.* [32], Elouennass *et al.* [33] *and* Ahmed *et al.* [34], as they reported susceptibility among ESβL-producers to carbapenem of 99.1%, 99.6% and 100%, respectively. Carbapenems are the drugs of choice; however, they are costly effective and the Carbapenem resistance among hospitalized patients is highly reported later on [35]. Therefore, the study of PROBIOTICS as a natural and a novel therapeutic option to be an alternative to antimicrobial drugs seems imperative*;* PROBIOTICS' is highly suggested to be more effective, non-toxic therapeutic option and recommended to be licensed for use in several countries. Then I had choose to evaluate the antibacterial effects of PROBIOTICS' culture extracts on these isolates that producing-ESβL and bearing the most prevalent gene [TEM-1] as an alternative to the antimicrobial drugs to be a novel therapeutic option against infections caused by these isolates. In this study, all isolates that detected as ESβL-producers, and bearing TEM-1 gene, were tested for susceptibility to PROBIOTICS' CULTURE EXTRACTS using the well-agar diffusion method and reducing the colony count activity technique.

All of the isolates being studied were susceptible to PROBIOTICS' CULTURE EXTRACTS; one strain of *Pseudomonas* shows the least susceptibility [12 mm of inhibition zone] and the least reducing colony count activity [by 50%]. The greatest activity against *E. coli* was [21 mm of inhibition zone] in one strain, while showing [100% reducing colony count activity] in 12 strains with zones of inhibition average in-between 17 and 21 mm. While the greater activity for the *proteus* was [20 mm of inhibition zone] for two strains, and shows [100% reducing colony count activity] for 3 strains with zones of inhibition average in-between 18 and 20 mm. The greater activity for *Klebsiella* was [20 mm of inhibition zone] for one strain and shows [100% reducing colony count activity] for 6 strains with zones of inhibition average in-between 17 and 20 mm. This relatively agreed with El-Mokhtar *et al.* [36] who reported susceptibility of *Klebsiella* to PROBIOTICS' filtrates with zones of inhibition average in-between 12.2 and 21.1 mm and 11.3 and 16.1mm for susceptibility of *pseudomonas.* While Heshmatipour *et al.* [37] reported the susceptibility of *E. coli* with zones of inhibition average in-between [14 and 20 mm] and Shaaban *et al.* [38] who reported the greater activity against multi-drug resistant *proteus* with 21 mm zones of inhibition. In addition to Chen *et al.* [39] who reported the greatest activity against 21 strains of carbapenem-resistant *E. coli* and for 9 strains of carbapenem-resistant *k. pneumonia* with zones of inhibition greater than 15 mm.

Conclusion: PROBIOTICS' CULTURE EXTRACTS revealed excellent activity against ESβL-producing microorganisms that bearing TEM-1 gene. So, these extracts may be considered safe therapeutic promising options to treat such infections and recommended to be licensed in a topical form of a gel solution in pharmacotherapy.

Financial and non-financial relationships and activities of interest

None to disclose

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