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Antibiotic resistance profile of local thermophilic *Bacillus licheniformis* isolated from Maysan province soil

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Abstract

The key concern for public health is that bacterial strains isolated from various ecosystems are immune to antibiotics used in human medicine, thus dramatically limiting therapeutic options and threatening the lives of infected people. The present study aims to reveal the antibiotics profile of fiftysix isolates of local thermophilic *Bacillus licheniformis* isolated from different environmental soil sites in Maysan city, Iraq. The antimicrobial agent resistance profile of *B. licheniformis* isolates was performed using the disc diffusion assay according to Kirby-Bauer susceptibility test protocol. The results showed that isolates were resistance against cefepime (n=56; 100%), amoxicillin (n=13; 23.3%) and ampicillin (n=52; 92.9%); and intermediate (n=56; 100%) against cephalothin and naldixic acid. The percentage resistance was low for aztreonam (n=4; 7%), chloramphenicol (n=3; 5%), clotrimazole (n=6; 10%), novobiocin (n=2; 3.5%) and ticarcillin (n=3; 5%). On the other hand, all isolates were sensitive (n=56; 100%) towards the following antibiotics: amikacin, ceftazidime, ciprofloxacin, clindamycin, imipenem, netilmycin, gentamicin, nitrofurantion, rifampin, trimethoprim and vancomycin. The results of this study suggest that the Iraqi thermophilic *B. licheniformis* isolates are variable in their susceptibility towards the standards antimicrobial agents. Furthermore, the presence of cefepime, amoxicillin, ampicillin, cephalothin and naldixic resistant isolates of *B. licheniformis* in Iraqi soils is of concern about how resistance could spread to other bacteria, and ultimately to humans.

Keywords: antibiotic resistance, *Bacillus licheniformis,* Iraqi soils, Maysan, thermophilic

Introduction

Bacillus licheniformis named by Weigmann 1898, is a Gram positive facultative, anaerobic, rod shaped and endospore-forming bacterium. The strains of this species are Widely distributed throughout the ecosystem as a saprophytic, where harsh conditions survive in the form of highly resistant endospores and belong to the *Bacillus subtilis* group (Garrity et al., 2009; Logan & Vos, 2015). Because of its ability to grow in a thermal environment like a hot spring, the bacterium is known to be facultative thermophilic or thermotolerant microorganism (Rodríguez-Lozano et al., 2010; Fritze, 2004).

It has generation time of 22min at 55°C compared to 41min at 37°C (Bischoff et al., 2006). *B. licheniformis* has been known to produce diverse substances that are useful in industries such as α-amylase, β-galactosidase, alkaline protease, anti-biofilm, and keratinase (Lin et al., 1992; Declerck et al., 2002; Sellami-Kamoun et al., 2008; Juajun et al., 2011; Sayem et al., 2011). While generally considered safe, there have been reports of occasional cases of *Bacillus licheniformis*-associated systemic infections in humans (Santini et al., 1995). However, without prior lesions it has no potential to penetrate the body 's outer barriers. However, abortions in sheep and cattle have been documented, and it has been demonstrated experimentally that *Bacillus licheniformis* is capable of infecting bovine placenta (Syrjälä et al., 2007; Agerholm et al., 1999).

Reported cases have suggested that lichenysin is produced in most strains of *B. licheniformis*. Bacillus licheniformis-associated food poisoning lichenysin has been characterized by a relatively short incubation period (2–14h) and a high dose of infection (> 10^5 $CFUg^{-1}$) accompanied by mild gastrointestinal symptoms lasting 6–24h (Salkinoja-Salonen et al., 1999; Madslien et al., 2013). Lichenysin is an important cyclic lipopeptide against

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the yeast (Noudeh et al., 2010; Kashid & Ghosh, 2010). Diarrhea is caused by food poisoning, but in half of the confirmed cases, vomiting occurs (Ara et al., 2007). One case of fatal illness associated with infant milk powder infected with *Bacillus licheniformis* has been reported (Mikkola et al., 2000; Salkinoja-Salonen et al., 1999).

In the past few years, the emergence of antibiotic resistance among bacteria has increased and its possible public health consequences have resulted in enhanced bacterial resistance surveillance in many countries. *Bacillus licheniformis* strains have received relatively little recognition for their antibiotic resistance; this may be due to their role as non-pathogenic species. Bacilli resistance to various antibiotics has been previously established, and it has been shown that species can be classified in theory on the basis of the results of their susceptibility tests (Reva et al.,1995).

The MIC test for some antibiotics was determined for strains of *Bacillus licheniformis* which were isolated from starters for the production of Sudanese bread. The MIC test was susceptible to tetracycline (8.0 mg / litre), vancomycin (4.0 mg / litre), and gentamicin (4.0 mg / litre), but was streptomycin resistant (Adimpong et al., 2012). Moreover, disc diffusion assay was reported for antibiotic resistance profiles in the genus of Bacillus particularly for *B. cereus* (Gao et al., 2018; Fiedler et al., 2019; Park et al., 2020), in contrast very few was reported for *B. licheniformis* (Jeong et al., 2017; Meneghetti et al., 2018). Because of the limitation of data which is available about the resistance of *B. licheniformis* and to provide a guidance value to clinicians encountering possibly *B. licheniformis* infections; this study was aimed to detect the antibiotic resistance profile of local thermophilic *B. licheniformis* isolated from soil in Maysan Province, Iraq.

Material and Methods

Fifty six isolates of thermophilic *Bacillus licheniformis* that isolated and confirmed from the previous study (Banoon & Ali, 2018) were included in this study. In brief, two grams of soil were added to 10 ml distilled water and mixed well, after a while 0.25 ml from soil suspension was transferred to 25 ml of Lauria broth medium and incubated at 55°C for 18 hours. Series of dilutions up to 10^{-3} were prepared for each sample. Each dilution was cultured on Lauria agar medium and incubated at 55°C for 18 hours. Upon growing, one colony was picked up from fresh overnight agar plates then cultured on a fresh Lauria agar medium and incubated at 65°C for confirming the tolerance of high temperatures (as thermophilic).

For the identification of *B. licheniformis* isolates, VITEK-2 compact system using BCL cards and *gyrase B* gene were used. The antibiotic-resistant of *B. licheniformis* strains was conducted using the disc diffusion method (Bauer et al., 1966). A pure culture of *B. licheniformis* was prepared by adding a growth from an isolated colony to 5 ml of sterile normal saline in a cell density equivalent to McFarland tube turbidity No. (0.5) which is approximately equal to a density of $1.5x10^8$ CFU/ml of bacterial cells.

A sterile cotton swab was used to Adding -from bacterial suspension to be streaked on Muller-Hinton medium. Twenty seven antibiotic discs (Al-Razi, Iraq) were tested, including amikacin (AMK, 30 µg/disc), amoxicillin (AMX, 25 µg/disc), amoxicillin-clavulanic acid (AMC, 20 µg/10 µg/disc), ampicillin (AMP, 10 µg/disc), aztreonam (ATM, 30 µg/disc), cefepime (FEP, 30 µg/disc), ceftazidime (CAZ, 30 µg/disc), cephalothin (CEF, 30 µg/disc), chloramphenicol (CHL, 30 µg/disc), ciprofloxacin (CIP, 5 µg/disc), clindamycin (CLI, 2 µg/disc), clotrimazole (CC, 10 µg/disc), erythromycin (ERY, 15 µg/disc), gentamicin (GEN, 10 µg/disc), imipenem (IPM, 10 µg/disc), kanamycin (KAN, 30 µg/disc), nalidixic acid (NAL, 30 µg/disc), netilmycin (NET, 30 µg/disc), nitrofurantoin (NIT, 300 µg/disc), novobiocin (NV, 30 µg/disc), oxacillin (OXA, 1 µg/disc), piperacillin (PIP, 100 µg/disc), rifampin (RIF, 5 µg/disc), ticarcillin (TIC, 75 µg/disc), ticarcillin-clavulanic acid (TIM, 75 µg/10 µg/disc), trimethoprim (TMP, 5 µg/disc) and vancomycin (VAN, 30 µg/disc). The discs were placed on the surface of the medium at evenly spaced intervals with flaming sterile forceps. The plates were incubated at 37 °C for 18 hr. For each antibiotic the inhibition zone was measured using a ruler.

The presence of a clear zone around the disk has been the index of antibiotic sensitivity. The antibiotic sensitivity test results were determined by the diameter of the inhibition zone (Barry, 1986; Çetin & Gurler, 1989).

The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the strains collected were resistant to that antibiotic test.

Results and Discussion

All isolated *B. licheniformis* strains were tested for their antibiotic resistance against a set of antimicrobial agents using Kirby-Bauer disk diffusion method. The Figure (1) showed that all strains were highly sensitive (n=56; 100 %) towards the following antibiotics: amikacin, ceftazidime, ciprofloxacin, clindamycin, imipenem, netilmycin, gentamicin, nitrofurantion, rifampin, trimethoprim and vancomycin. However, these results were compatible with studies of (Weber et al., 1988; De Clerck & De Vos, 2004). In contrast, all isolates showed resistance to cefepime (n=56; 100 %) and intermediate (n=56; 100 %) to cephalothin and naldixic acid.

Others antibacterial agents were varied in their effects in different ways where the isolates showed resistance against amoxicillin (n=13; 23.3%) and ampicillin (n=52; 92.9%). Moreover, the results showed that the percentage resistance was low for aztreonam (n=4; 7%), chloramphenicol (n=3; 5%), clotrimazole (n=6; 10%), novobiocin (n=2; 3.5%) and ticarcillin (n=3; 5%).

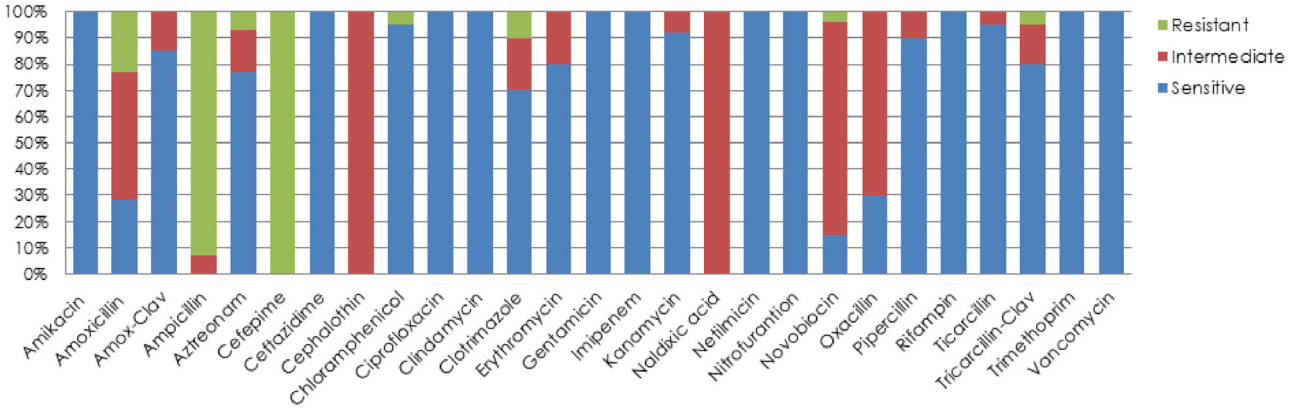

Figure 1. Antibiotic sensitivity profile of *Bacillus licheniformis* based on inhibition zone diameter (mm); (green) resistance, (red) intermediate, (blue) sensitive.

There are few studies that showed the sensitivity of *Bacillus* species to antibiotics even though they are related to the genus taxonomy. Banerjee et al., (2007) has noticed that *B. licheniformis* strains were 100% susceptible to oxytetracycline, gentamicin and chloramphenicol and 40% were susceptible to bacitracin. Penicillin G has been reported to be less active against *B. licheniformis* (Weber et al., 1988).

A study carried out by Abdel-Shakour & Roushdy (2010) of Thermo tolerant soil isolate of *Bacillus cereus* BC2, found that the strain was resistant to the Ampicillin and Amoxycillin while sensitive to the Erythromycin, Amikacin, Chloramphenicol, and Kanamycin. Another study done by Sarker et al., (2010) for *Bacillus thuringiensis* strains from dump soil showed that the strains were sensitive to erythromycin, chloramphenicol, kanamycin, ampicillin, and vancomycin. On the other hand, when checked by the method of disc diffusion on the nutrient agar plate, the strains showed resistance to amoxicillin, as verified by the method of antibiotic spread plate. Previous studies showed that Vancomycin is bactericidal at or near the same concentration at which it is bacteriostatic (Weber et al., 1988). Almost all strains in our study were resistant to ampicillin (n=52; 92.9 %), this was in agreement with a previous study (Andrews & Wise, 2002).

The Chloramphenicol inhibits protein synthesis by binding to the ribosomal subunit of the 50s and blocking the peptidyltransferase reaction. By binding to the ribosome and preventing translocation, gentamycin inhibits protein synthesis. Rifampin prevents transcription initiation by binding RNA polymerase to the ß subunit of the prokaryotes (Harvey & Ferrier, 2011).

Kanamycin prevents protein synthesis by binding to and preventing translocation from the ribosomal subunit of the 30s. Timentin is a combination of the penicillin derivative Ticarcillin and the B-lactamase inhibitor, clavulanic acid the exogenous ß-lactamase is inactivated by the clavulanic acid, which enables Ticarcillin to prevent the growth of any sensitive cells in the population. Trimethoprim is an inhibitor of the reductase in bacterial dihydrofolic acid (Zähner & Maas, 1972). In the study carried out by (Ozkocaman et al., 2006), the author showed that *B. licheniformis* isolates were resistant to Clindamycin, penicillin and other B-lactam agents, whilst aztreonam, Imipenem, cefepime, ciprofloxacin, meropenem, levofloxacin, ofloxacin, tetracycline and Vancomycin remained fully active.

A study was done by Bautista & Teves (2013) for *B. thuringiensis,* which isolated from vermicast a loam soil which considered as non-pathogenic for humans and used extensively for pest control, was found to be resistant to the β-lactams (amoxicillin and ampicillin). Ampicillin and amoxicillin belong closely to a class of antibiotics called penicillins which are used to treat bacterial infections. Such antibiotics have a common mechanism of action by preventing bacterial cells from multiplying because the walls that surround them are not forming. Although the results of this study have illustrated susceptibility to the most tested antimicrobial agents, several recent studies have been shown increasing in resistance of *B. licheniformis* strains towards antibiotics (Adimpong et al., 2012; Agersø et al., 2018; Agersø et al., 2019; Moore et al., 2019).

Cefepime is a broad-spectrum β-lactam antibiotic that has been introduced in the market for the treatment of serious bacterial infections particularly for the

treatment of Gram-negative bacteria (Gupta et al., 2006; Donà et al., 2019). Surprisingly, all the isolates in our study were resistant toward cefepime. However, some Gram-positive bacteria including *Streptococcus pneumoniae*, *Streptococcus thermophilus, Enterococcus faecium*, and *Staphylococcus aureus* have developed several mechanisms for β-lactam resistance (Fisher & Mobashery, 2016; Lemonidis et al., 2019; Aldujaili, & Banoon, 2020).

In the genus of Bacillus resistance towards β-lactam antibiotics was also reported. Fenselau et al. (2008) reported that *B. cereus* 5/B line (ATCC 13061) was resistant towards β-lactam antibiotics. In *B. licheniformis* mechanism of resistance has been observed by the production of BlaP b-lactamase (Vandevenne et al., 2007) and the peptidoglycan fragment that triggers β-lactam resistance (Amoroso et al., 2012). In addition, the resistance found to cephalothin and nalidixic acid is not surprising as it coincides to what has been found in several studies (Barry & Jones, 1984; Akinbowale et al., 2006; Crowe-McAuliffe et al., 2018). In a study carried out by Shale & Malebo (2011), the authors found that 97% of the *B. cereus* isolates (n=90) were resistant to nalidixic acid.

Conclusion

The study concludes the high prevalence of *B. licheniformis* and its antibiotic resistance characteristics in Iraqi soils. The resistance of isolates to cefepime, cephalothin, naldixic, amoxicillin, ampicillin, aztreonam, chloramphenicol, clotrimazole, novobiocin and ticarcillin has shown that such antibiotics should not be used in human therapy without prior determination of susceptibility.

Moreover, where the antibiotic resistance of the bacterial strains usually results from chromosome mutations or from the acquisition of resistance plasmids (Inglis, 1999; Balakrishnan et al., 2003), the use of antibiotics for humans needs to be regulated strongly to minimize the opportunity for microorganisms to develop resistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.