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Detection of Virulence Genes in Some of *Bacillus sp* Isolated from Camel Urine Using PCR Analysis

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Abstract **Original Research Article**

Bacillus is a genus that contains both environmental and pathogenic species that can cause food poisoning in humans as a result of consuming foods containing toxins or bacteria. In this study, the 16S rRNA gene of the bacteria was sequenced to identify bacteria that were isolated from camel urine. Detected the Bacillus enterotoxins that encoding by three virulence genes; nonhemolytic enterotoxin (NHE) gene, cytotoxin K (CytK) gene and hemolytic enterotoxin (HBL) gene hblD. *Bacillus sp* isolates were examined for detection *Bacillus enterotoxins* (NheA,hblD, and CytK) by PCR. NheA gene was detected in two of 78 examined samples 2.5% while, cytK gene was detected in 1.2 % of the examined samples. On the other hand, hblD gene failed to be detected.

Keywords: Virulence genes, enterotoxins, PCR, 16SrRNA, Bacillus.

INTRODUCTION

Camel urine has been considered a "miraculous" Prophetic Medicine since ancient times and was commonly used in the pre-Islamic era (O'haj *et al*., 1993). Camel urine contains metabolites that exhibit beneficial pharmacological properties similar to those of antibacterial and antifungal (Al-Bashan, 2011). Microbial virulence factors are a group of chemicals produced by pathogenic bacteria that enhance their potential to cause disease. Toxins, enzymes, and exopolysaccharides are examples of secreted products, as well as cell surface structures such as capsules, lipopolysaccharides, glycol, and lipoproteins. Intracellular alterations in metabolic regulatory networks, which are mediated by protein sensors/regulators and non-coding regulatory RNAs, have also been linked to virulence. In addition, some released microbial compounds have the ability to infiltrate the host cell and modify its machinery, aiding infection success. The discovery of novel therapeutic compounds and tactics to battle microbial infections requires a thorough understanding of the biology of microbial pathogens and their virulence factors at the molecular level (Leitão, 2020 & Denzer *et al*., 2020).

Bacillus is a spore-forming resistant to unfavorable external conditions and a Gram-positive aerobic bacteria genus (Logan and Devos, 2009 & Abd El Tawab *et al*., 2019).

Several species in this family are non-pathogenic, easy-to-grow, and release enzymes including proteases, amylases, and cellulases that are helpful in a variety of industrial applications (Arbige *et al*., 1993). Usually, the diarrheal condition appears 8 to 16 hours after consuming tainted food (Park *et al*., 2009). Foods tainted with *B. cereus* may increase the chance of contracting a food-borne illness. Control procedures during food preparation or after processing are therefore necessary to ensure that consumption is safe (Sornchuer, & Tiengtip, 2021).

Due to the release of heat-labile enterotoxins, the bacterium is typically linked to food poisoning and diarrhea (Granum, 1994; Forghani, 2015 and Tharwat, 2020).

Hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), cytolysin K (CytK), and enterotoxin FM (EntFM) are the enterotoxins that cause diarrhea (Kim *et al*., 2011; Hwang and Park, 2015). The Hbl toxin, is composed of the binding component B, two lytic proteins (L1 and L2), and hemolysis, cytotoxicity, dermonecrosis, and increased vascular permeability. The proteins that make up the toxins (CytK and EntFM) single-component proteins. HBL is a three- component

toxin, that is expressed by hblD and hblC genes respectively, and the binding component B is expressed by the hblA gene. The activation of the toxin depends on each of the three elements being present (Lindback and Granum, 2006).

The cytotoxin K (cytK), which is known as the primary virulence factor in diarrhea, is extremely cytotoxic and has the potential to result in hemolysis and necrosis (Lund *et al.*, 2000). Therefore, this study was performed to determine the prevalence, and incidence of enterotoxin genes (cytK, nheA, and hblD)-carrying Bacillus sp isolates from camel urine at Taif city, Kingdom of Saudi Arabia.

MATERIALS & METHODS

Samples Collection

Using sterile containers the urine samples were collected from (camels that graze in open places in Taif). The samples were collected with the help of camel patrons after cleaning and sanitizing the skin of the camels. While camel patrons used sterile gloves and scrubbed the camel skin with alcohol 70%.

The urine samples were collected in 30 mL sterile tubes and the samples were transferred to the lab within 4 hours of collection under cooling.

Isolation and Identification of *Bacillus sp*. group

Fresh urine samples were transferred immediately to the lab after collection and serial dilutions of the samples by adding 1 ml of each sample were transferred in the tube containing 9 ml of sterile distilled water (10-1) to (10-5).

Serial dilutions (10-1 — 10^5) were prepared for both samples taking the initial dilution as 10-1. From the dilution range of 10^2, 10^3, 10^4, and 10^5, 0.1 mL of the sample was inoculated in nutrient agar (NA), MacConkey agar, and sheep blood agar using spread plate technique (Kumar *et al*., 2012; Eze, 2009; Sherman *et al.*, 2009). The inoculated plates were incubated at 37^0 C for 24 hrs (Eze, 2009). After incubation individual colonies were isolated in pure culture using the streak plate technique (Eze, 2009; Sherman *et al*., 2009).

Molecular Identification of *Bacillus sp.* using 16S rRNA

DNA Extraction

Total genomic DNA was isolated according to Azcárate-Peril and Raya (2001) with some modification, where 200 µl of TES buffer and 20 µl of lysozyme (10 mg/ml) were mixed with a suitable amount of bacterial pellets of overnight culture. The mixture was incubated at 37^o C for 20 min in a water bath.

20 µl of proteinase K (10 mg/ml) was added to each sample and then incubated at 37^oC for 20 min in a water bath. The mixture was transferred to an ice bath for 5 min then 250 µl of 4M sodium acetate was added. 250 µl of chloroform: isoamyl (24:1) was added then the mixture was stirred between fingers to mix it then centrifuged at 13000 rpm/ 2 min. the upper zone was transferred with care to a new clean Eppendorf and 3/4 or 1 v/v of isopropanol was added then reserved at -20 overnight. The next day, the solution was centrifuged at 13000 rpm/2 min then the liquid zone was totally discarded, and DNA was left to be dried at room temperature and then resuspended with 50 µl of distilled water. 10 µl of isolated DNA was loaded in 0.5% agarose gel in 1x of TBE buffer at 100 V for 45 min and stained with ethidium bromide.

DNA Concentration

DNA concentration and the quality in terms of 260:280 ratios in the samples were determined using Nanodrop (NanoDrop 8000 UV-Vis Spectrophotometer, Thermo Scientific, USA).

16S rRNA Gene Amplification Sequencing and Phylogenetic Analysis

Primers

The 16S rRNA gene was amplified using universal primers, 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) (for all isolates), 1492R (5´-AAGGAGGTGATCCAGCCGCA-3´).

PCR Protocols

For the amplification of the DNA, a Thermo Scientific PCR master mix was used following the manufacturer's guidelines. Amplification was done in a thermocycler (Mastercycler® Gradient, Eppendorf, Hamburg, Germany) at 94 ºC for 5 min, followed by 35 cycles of 30 s at 94 ºC, 30 s at 57 ºC and 30 s at 72 ºC, with a final extension at 72 ºC for 10 min.

Analysis of Amplified PCR Product

A 3µl aliquot of each PCR amplicon was electrophoresed on a 1 % agarose gel containing ethidium bromide in 1X Tris-Acetate-EDTA (TAE) buffer at 120 V for 40 min and visualized under a UV transilluminator (BioDoc-IT system, Japan).

Amplified PCR Amplicons' Sequencing

Amplified products were purified using QIAquick PCR purification kit (Promega, Madison, WI, USA) and sequenced using the Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Forster City, CA, USA) in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

The sequences were edited by the SnapGene Viewer software version manually and then, compared with the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTN search, and reference sequences were retrieved to perform phylogenetic analyses. Phylogenetic trees were constructed using the MEGA available on the NCBI website.

Preparation of the Acrylamide gel

A. Gel cast was cleaned and mounted properly (0.75mm spacers).
B. Following components were added and mixed together to a final volume of 5mL, 1.3 mL acrylamide: bisacrylamide solution (29:1), 2.6 mL water, 500 µL 10X TBE solution, 35 µL of 10% Ammonium Persulfate (w/v) and 2µL TEMED.
C. The acrylamide solution was pipetted between the casting plates.
D. The comb was inserted carefully to avoid bubbles and waited for 30 minutes. Finally, the comb was removed from the gel carefully and the gel was mounted to the electrophoretic assembly. The running buffer (1X TBE) was added to the assembly and samples and DNA marker was loaded on the gel with the help of a pipette carefully. Electrophoresis was performed at 80 V for 60 minutes. Gels were stained with ethidium bromide and were visualized under Gel-Doc system (Bio-Rad, Universal Hood II, USA).

Detection of Virulence Factors of *Bacillus sp* isolates

To detection of Bacillus enterotoxins, three virulence genes; nonhemolytic enterotoxin (*NHE*) gene *NheA* and cytotoxin K (*CytK*) gene, and hemolytic enterotoxin (*HBL*) gene *HblD*.

a. Extraction of DNA

All isolates of *Bacillus sp., that was* isolated from different urine samples were grown in 5 mL nutrient broth with shaking for 24 h at 37 °C and collected**.**

The genomic DNA of bacteria isolates was extracted according to the method described by (Tabrez & Hiraishi, 2002).

The primers for PCR amplification of the virulence genes were described in (Table 2). A master mix was used following the manufacturer's guidelines. Amplification was done in a thermocycler (Mastercycler® Gradient, Eppendorf, Hamburg, Germany). Pcr master mix component used for PCR reaction for detection of virulence genes of *Bacillus sp.,* were described in (Table 1).

Cycling Conditions of the Primers during PCR

PCR was achieved to detect three enterotoxigenic encoding endotoxins genes nheA, hblD, and cytK genes. In the PCR study, sterile MilliQ water was used as a negative control (Ehling-Schulz *et al.,* 2006; Das *et al.,* 2013).

PCR Conditions

Amplification was done in a thermocycler (Mastercycler® Gradient, Eppendorf, Hamburg, Germany) at 94 ºC for 2 min, followed by 35 cycles of 30 s at 94 ºC, 45 s at 55 ºC and 2 min at 72 ºC, with a final extension at 72 ºC for 2 min.

PCR fragments were examined using gel electrophoresis for presence and proper size in comparison to a positive control (Sombrook *et al*., 1989).

Table 1: Pcr master mix component used for PCR reaction for detection of virulence genes of Bacillus sp.

Component	**Volume**
Emerald Amp GT PCR mastermix (2x premix)	12.5µl
PCR grade water	10.5µl
Forward primer(20 pmol)	0.5µl
Reverse primer (20 pmol)	0.5µl
Template DNA	1µl
Total	**25µ**

Table 2: Temperature and timing setting for the PCR experiment

Target gene	Primer name	Oligonucleotide sequence(5´-3´)	Product size(bp)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	1484	Deng *et al.*,(2015)
	1492R	AAGGAGGTGATCCAGCCGCA		
nheA	nheA 344S	TACGCTAAGGAGGGGCA	499	Hansen and Hendriksen(2001)
	nheA 843A	GTTTTTATTGCTTCATCGGCT		
hblD	HD F	ACCGGTAACACTATTCATGC	829	Gulnebretier *et al.*,(2002)
	HD R	GAGTCCATATGCTTAGATGC		
CytK	Cytk-F	ACAGATATCGGGTCAAAATGC	421	Ehling-Schulz *et al.*,(2006)
	Cytk-R	GAACTGGATAACTGGGTTGGA		

RESULTS

Prevalence of *Bacillus sp in Camel Urine Samples*

Members of the genus Bacillus were the most prevalent species, present in over 64% of the samples, *namely, B. velezensis, B. tropicus, B. haynesii, B. subtilis, B. albus, B. amyloliquefaciens terium, B. sonorensis, B. aryabhattai, B. amyloliquefaciens, B. flexus,B. nitratireducens and B. inaquosorum.*

Detection of Enterotoxin Genes

PCR Results

The result obtained using agarose gel electrophoresis of PCR of virulence genes, HblD gene (829 bp), NheA (499 bp), and Cytk gene (421bp) for characterization of virulence genes of *Bacillus sp* isolated from different camel urine samples showed that in (Table 3). NheA gene (499bp) was detected in two of 78 examined samples 2.5% while, *CytK* gene (421bp) was detected in 1.2 % of the examined samples (fig. 1). On the other hand, *HblD* gene failed to be detected. The result of the samples was tested at five different temperatures of annealing (gradient temperature 52-56 C°), the best band clarity of *CytK* gene of samples at 56C° (fig. 2).

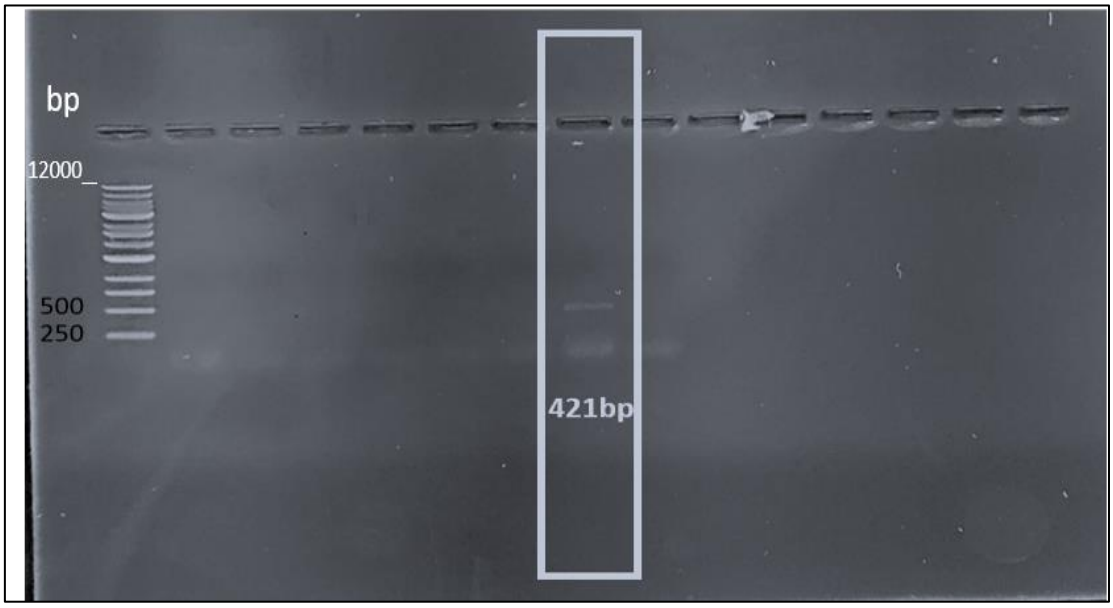

Fig. 1: Results of PCR amplification of CytK gene of Bacillus sp isolated from different camel urine samples. Bacillus amyloliquefaciens positive samples at 421 bp and 56°C. Genesta 1Kb DNA Ladder, 5ul/laneEB staining,1.0% agarose gel

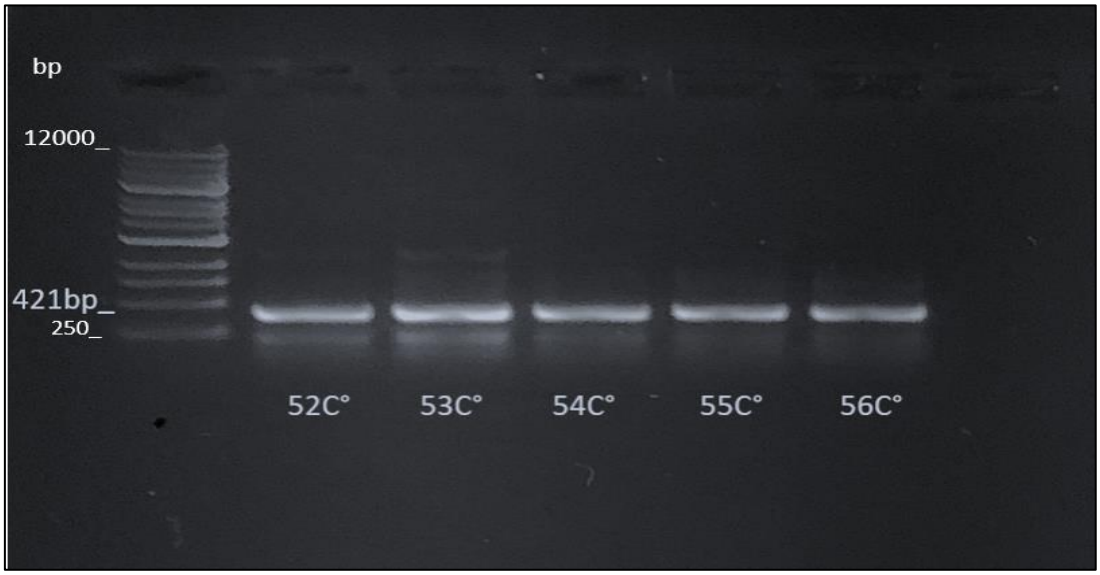

Fig.2 : Annealing temperature gradient on the band clarity of (cytK) gene , of Bacillus amyloliquefaciens samples isolated from camel urine,the best temperature at 56C°.Genesta 1Kb DNA Ladder, 5ul/laneEB staining,1% agarose gel

Table 3: Distribution of virulence factors among isolates of *Bacillus sp*. Obtained from camel urine samples

Virulence gene / *Bacillus sp*	CytK	NheA	HblD
Bacillus velezensis	-	-	-
***Bacillus subtilis* spp.**	-	-	-
Bacillus sonorensis	-	-	-
Bacillus haynesii	-	-	-
Bacillus aryabhattai	-	-	-
Bacillus amyloliquefaciens	+	+	-
Bacillus tropicus	-	-	-
Bacillus inaquosorum	-	-	-
Bacillus nitratireducens	-	-	-
Bacillus flexus	-	-	-
Bacillus albus	-	+	-

DISCUSSION

Bacillus species were found to be the most numerous in more than 68% of the samples, according to 16S rRNA gene sequence analysis. *Bacillus spp*. spores or vegetative forms have been employed as probiotics, and they have a high level of stability in the presence of heat, gastrointestinal environs, and moisture (Lee, *et al*.,2019).

All strains of *Bacillus sp* (78 samples) were tested for the presence of the Bacillus enterotoxins that encoding to three virulence genes; nonhemolytic enterotoxin (*NHE*) gene *NheA*, cytotoxin K (*CytK*) gene, and hemolytic enterotoxin (*HBL*) gene *HblD*.

The presence of the *NheA* gene was discovered in two (2.5%) strains *Bacillus albus* and *Bacillus amyloliquefaciens*, while *CytK* gene was detected in *Bacillus amyloliquefaciens* also (1.2%). However, *HblD* gene was not detected.

Previously has been discovered that the hemolytic enterotoxin (*HBL*) gene (*HblD*) has been found in 68 % of *B. cereus*, while the nonhemolytic enterotoxin (*NHE*) gene (*NheA*, was found in 71% of isolates(Al-Khatib *et al*. 2007).

Nhe gene and *Cytk* gene were present in 100% of *B. cereus* isolates from various meat sources, whereas *Hbl*D gene was found in 10% of isolates it is a small percentage compared to another gene in a previous report (Abd El Tawab, *et al*. 2020), this is somewhat similar to the results of this study in terms of the most bacillus prevalent virulence genes NheA, CytK were (2.5% & 1.2%) respectively.

CONCLUSION

The toxin gene distribution of Bacillus bacteria was analyzed with the aid of this work, which should be helpful to understand the potential toxigenicity of Bacillus in camel urine. Finally, we recommend more studies and detection of the presence of antibiotic-resistance genes and the use of more advanced techniques for a wide range of bacterial species to detect the presence of any potential risks of using camel urine for therapeutic purposes.

Competing interests: No competing interest exists

Authors' contributions: All authors contributed to this work.

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