CATRINA (2016), 15 (1): 51-57

Identification of *Bacillus Thuringiensis* Isolated from Different Sources by Biolog GEN III System and Scanning Electron Microscopy

Hend Abdulhameed Hamedo
Faculty of Science, El Arish University, El Arish, Egypt

ABSTRACT

The present study aims to isolate and characterize *Bacillus thuringiensis* (*Bt*) strains isolated from different sources. These strains were subjected to morphological and biochemical characterization. The most active five isolates were presumptively confirmed as *Bt* strains. They were resistant to 8% NaCl, unable to survive at 50°C, motile and had haemolytic activity. The 5 isolates showed positive reaction to catalase test, methyl red test, arginine decarboxylation, citrate utilization and gave negative reactions to the oxidase test and indole test. All of them were also able to ferment glucose, sucrose, maltose, fructose, raffinose, dextrin, glycerol, pectin and gelatine whereas they could not ferment mannitol, arabitol, rhamnose, lactose, mannose or sorbitol. All isolates were Gram-positive and sensitive to Penicillin, Tobramycin, Bacitarcin and Amoxycillin/Clavulinic acid whereas they were resistant to Clindamycin, Nitrofuratoin, Candicidin and Chlramphenicol. The surface features of *Bt* spores were more clearly revealed with the scanning electron microscope. *Bt* spores have been described as cylindrical to ellipsoids in shape, surface smooth to very slightly ribbed and were covered with exosporium.
Key words: *Bacillus thuringiensis*, Biolog GEN III, Scanning electron microscopy.

INTRODUCTION

Bacillus thuringiensis (Bt) is an aerobic, gram-positive, endospore forming bacterium which during sporulation produces crystalline inclusions consisting of one or more insecticidal proteins known as delta-endotoxins. *Bt* is the most successful agent of microbial control discovered to date (Fakruddin *et al*., 2012; Halima *et al.,* 2006). *Bt* is prevalent not only in soil but also in many other habitats worldwide (Alam *et al.,* 2008).

Biolog III Gen recently introduced as an automated system designed to identify hundreds species or groups of bacteria within 4 to 24 hrs. Their data base includes environmental taxa that are not included in the data bases of the other commercial systems (Wragg *et al*., 2014). The biology system is based on tests for the oxidation of 95 substrates in a 96-well microtiter plate. Each well contains a redox dye, tetrazolium violet that permits colorimetric determination of increased respiration that occurs when cells are oxidizing a carbon source. Reactions are read after 4 hrs and/or after overnight incubation (Bochner, 2008).

The test results may be read by eye and recorded manually or read with an automated plate reader. The large number of characters makes this system particularly suitable for reference or research laboratories. However, with more biochemically reactive taxa, the system displayed a valuable capacity to identify species which were otherwise difficult to identify and, in some cases, a capability to detect relationships between epidemiologically-related strains (Jánosi *et al*., 2009; Lanka *et al*., 2010).

In its most recent manifestation, GEN III, a single plate was designed to cover both Gram-negative and Gram-positive aerobic taxa, reducing costs and improving flexibility. Scanning Electron Microscopy (SEM) is a powerful method for the investigation of surface structures of microorganisms. This technique provides a large depth of field, which means, the area of the sample that can be viewed in focus at the same time is actually quite large (Stadtländer, 2007). SEM has also the advantage that the range of magnification is relatively wide allowing the investigator to easily focus in an area of interest on a specimen that was initially scanned at a lower magnification. This study was designed to identify some bacterial isolates from different sources by using two methods: Biolog GEN III system and scanning electron microscopy.

MATERIALS AND METHODS

Isolation of strains

Bacterial isolates used in this study were isolated from different sources such as pasteurized full milk from market (Fm), rain water (R) and soil sample (S). The strains were grown on nutrient agar medium (NA) containing the following (g/l): glucose, 5.0; peptone, 5.0; sodium chloride, 5.0; beef extract, 3.0 and agar, 20.0. Five isolates designated as *Bacillus* spp. exhibited relatively faster and highly growth rates than the rest were picked and chosen for further study.

Morphological study

All the isolates were stained to observe Gram reaction. Cultures grown for approximately 5 days were examined microscopically to show the spore formation after staining with spore staining.

Antibiotic sensitivity assay

All strains were tested for antibiotic resistance using the standard agar disc diffusion technique (Aligiannis *et al*., 2001) on Mueller Hinton agar using commercial discs (Oxoid, UK). The antimicrobial agents used were Penicillin (10 µg), Tobramycin (10 µg), Clindamycin (20 µg), Nitrofuratoin (300 µg), Bacitarcin (10 µg), Chloramphenicol (30 µg), Candicidin (10 µg) and Amoxycillin /Clavulinic acid (30 µg). Susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (CLSI, 2006).

* *Corresponding author: hend_hamedo@hotmail.com*

Hemolysis assays

For determination of Haemolytic activity, five bacterial strains were propagated on blood agar supplemented with 5% sheep erythrocytes and incubated at 37°C for 24 hrs. Colonies producing clear zones of haemolysis were recorded as haemolysin positive (Bodade *et al.,* 2009).

Starch hydrolysis

The ability to use starch as a sole source of carbon and energy was tested. Starch agar medium which composed of beef extract, soluble starch and agar was used. After inoculation at 35°C for 48 hrs clear zones indicated the hydrolysis of starch (Smibert and Noel, 1994).

Growth at different temperatures

Aliquots of Luria-Bertani (LB) broth (5 ml) which containing the following (g/l): tryptone, 10; yeast extract, 5; NaCl, 10; were inoculated with 50 µl (105-106 CFU/ml) of freshly grown (16 ± 2h) native *Bacillus* isolates and incubated at different temperatures (25, 38, 45 and 55°C) for 24 - 48 hrs. After incubation the tubes were observed for growth which was indicated by turbidity change.

Biochemical characterization

Tests were conducted to characterize the biochemistry of the isolates using Biolog GEN III system.

Biolog GEN III microplate system

A single colony selected and emulsified into inoculating fluid A (Biolog) for subsequent inoculation on to the MicroPlate test plate (Biolog) (Wragg, *et al*., 2014). The inoculum prepared to a specified transmittance using a turbidity meter, as specified in the user guide.

For each isolate, 100 µl of the cell suspension was inoculated into each well of the MicroPlate, using a multichannel pipette and incubated at 37°C for 20 hrs, either aerobically or in 7.5% CO_2, according to growth characteristics. Micro Plates read in the MicroStation semi-automated reader after 20 hrs and results interpreted by the identification system's software (GEN III database).

Scanning electron microscopy

Aliquots (5 ml) of freshly grown bacterial cultures (incubated for 16 ± 2 hrs at 37°C) were harvested, fixed, dehydrated and embedded essentially as described by Da Silva *et al*. (2010). Microscopic examination carried out using scanning electron microscope (Leo Electron Microscopy Ltd. Cambridge, UK).

Spore hydrophobicity

Spore hydrophobicity were determinate by adhesion to hexadecane (Smirnova *et al*., 2013).

RESULTS

The morphology of colony revealed that they smooth with an irregular shape. To test the sensitivity of the isolates, 8 commercial antibiotic discs were used. The results interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (table 1). Isolates showed high sensitive to Penicillin, Tobramycin, Bacitracin and Amoxycillin /Clavulinic acid while all had high resistance against Clindamycin, Nitrofuratoin, Candicidin and Chlramphenicol. Table (2) indicated that strains Fm1, Fm3 and S1 were found to have the ability to hydrolyse starch and use it as a sole carbon and energy source for growth, while the strains Fm2 and R1 have not this ability. On the other hand, all strains have haemolytic activities.

Table (1): Antibiotic sensitivity test of *Bacillus* strain.

Bacterial strains	P	Tb	Cd	Nf	B	C	Fr	Ac
Fm1	++	++	-	-	++	-	-	++
Fm2	++	++	-	-	++	-	-	++
Fm3	++	++	-	-	++	-	-	++
R1	++	++	-	-	++	-	-	++
S1	++	++	-	-	++	-	-	++

P: Penicillin10 ug, **Tb**:Tobramycin 10 ug, **Cd**: Clindamycin 2.0 ug, **Nf**: Nitrofuratoin 300 ug, **B**: Bacitarcin 10 ug, **C**: Chlramphenicol 30 ug, **Fr**, Candicidin (10 µg) and **Ac**:Amoxycillin/Clavulinic acid 30 ug.

Table (2): Results of starch hydrolysis and hemolytic activities test.

Bacterial strains	Hemolysis	Starch hydrolysis
Fm1	+	+
Fm2	+	-
Fm3	+	+
R1	+	-
S1	+	+

Morphological and biochemical characteristics of the bacterial strains

Gram stain indicated that all bacterial isolates rod shape; Gram-positive and motile (table 3). The 5 isolates showed positive reaction to catalase test, methyl red test, arginine decarboxylation and citrate utilization and negative reactions with the oxidase and indole tests. Isolates ferment glucose, sucrose, maltose, fructose, raffinose, dextrin, glycerol, pectin and gelatin whereas they could not break down mannitol, sorbitol, arabitol, rhamnose, lactose, mannose or sorbitol.

All isolates were unable to tolerate 50°C and represented high growth at 38°C. According to the biochemical identification by Biolog GEN III all strains were capable to grow at different pH degrees but the enhanced growth were at pH 6. The bacterial isolates able to grow at different salt concentrations were determined. The five strains were tolerant to different concentration of NaCl (1 - 8%), and could grow at low concentration of NaCl (1%).

Numerous studies showed that the morphological diversity of spores combined with the heterogeneity of their physicochemical and biological characteristics. In the present work, the spores of different isolates of *BT* were examined under a scanning electron microscopy (Fig. 1).

***Bacillus thuringiensis (Bt)* spore physical properties**

Bt spores described as oval, cylindrical to ellipsoids in shape, depending on the viewer, surface smooth to very slightly ribbed and were covered with exosporium then difficult to remove the vegetative cell walls.

All five *Bt* strains vary in length than width with average of 1.42 μm for length and 0.83 μm for width. Assessment of hydrophobicity of the spores of *BT* by adhesion to hexadecane revealed that hydrophobicity indices for the strains were ranging from 32 to 46%. From the figures there were many appendages appear on the spore surfaces.

Table (3): Morphological and biochemical characteristics of the bacterial strains

Test organisms	Fm1	Fm2	Fm3	R1	S1
Cell Morphology	Rod	Rod	Rod	Rod	Rod
Gram stain	+ve	+ve	+ve	+ve	+ve
Motility	Motile	Motile	Motile	Motile	Motile
catalase test	+	+	+	+	+
methyl red test	+	+	+	+	+
oxidase test	–	–	–	–	–
indole test	–	–	–	–	–
Utilization of sugars					
Arginine dehydrogenase	++	+	+	++	+
Citrate utilization	+++	++	++	++	+
D-Cellobiose	++	+	-	++	-
Glucose	+++	+++	+++	++	++
D-Mannitol	-	-	-	-	-
D-Sorbitol	-	-	-	-	-
D-Fructose	++	++	++	++	++
Sucrose	++	+++	+++	+++	+
D-Arabitol	-	-	-	-	-
L-Rhamnose	++	-	-	++	-
D-Galactose	+	-	-	++	++
Lactose	-	-	-	-	-
D-Maltose	+++	+++	+++	+++	++
D-Mannose	-	-	-	-	-
Inositol	++	-	-	++	++
D-Raffinose	++	+	+	++	+
D-Sorbitol	-	-	-	-	-
Dextrin	+++	+++	+++	++	++
Glycerol	+++	++	++	+++	+++
Pectin	+++	+++	++	++	+
Gelatin	+++	+++	+++	++	++
Formic acid	+++	++	+	+++	++
L-Lactic acid	+++	++	+++	+++	++
Acetic acid	++	+	+	++	+
Growth at different pH					
5	+	+	+	+	+
6	+++	+++	+++	+++	+++
Growth at different concentration of NaCl (%)					
1	+++	+++	+++	+++	+++
4	+	+	+	+	+
8	-	-	-	-	-
Growth at different Temperature(°C)					
25	-	-	-	-	-
38	+++	+++	+++	+++	+++
45	+	+	+	+	+
50	-	-	-	-	-

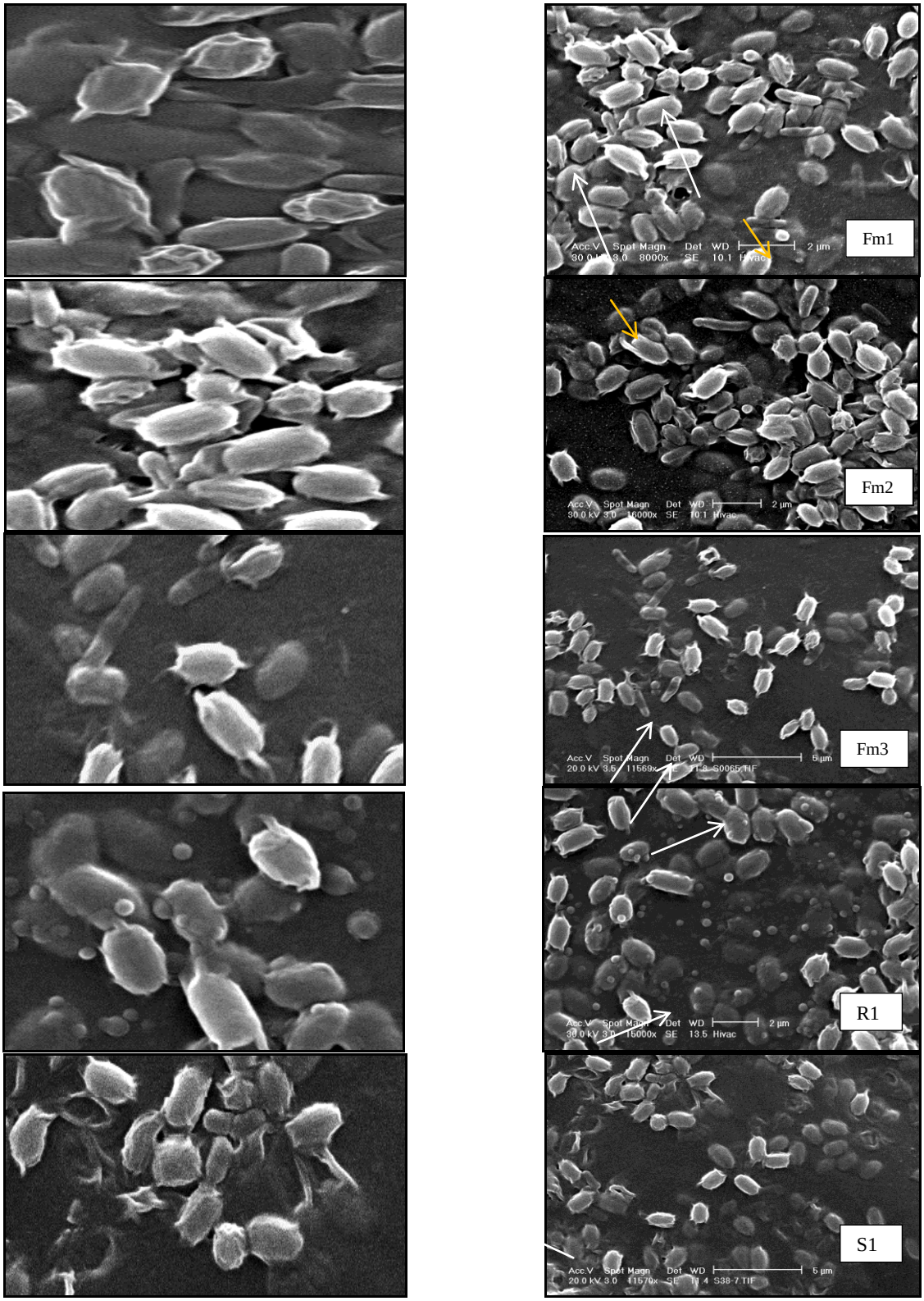

Figure (1): Scanning electron microscopy of *B. thuringiensis* isolates. (Fm1, 2, 3) isolates from pasteurized full milk, (R1) isolated from rain water and (S1) isolated from soil sample. The white arrows sign to the appendages from the spores, while the red arrow sign to the spore with folded exsporium, scale bar = 2 µm.

DISCCUSION

The traditional methods of bacterial identification are based on observation of either the morphology of single cells or colony characteristics. However, the adoption of newer and automated methods offers advantage in terms of rapid and reliable identification of bacterial species. Five strains identified as *Bacillus thuringiensis* (*Bt*)

were examined for their biochemical and morphological properties by Biolog GEN III and scanning electron microscope.

Three strains were able to hydrolyse starch. This test is used to differentiate bacteria based on their ability to hydrolyse starch with the enzyme α -amylase or oligo-l, 6-glucosidase. MacFaddin (2000) stated that organisms that produce and secrete the extracellular enzymes α -amylase and oligo-l, 6-glucosidase are able to hydrolyse starch by breaking the glycosidic linkages between the sugar subunits.

Haemolysis is indicated as clear zones around the colonies. The current results were in agreement with Bodade *et al.* (2009) who found that the majority of *Bt* isolates from soil samples with haemolytic ability.

Isolates fermented many types of carbohydrates. These results were similar to the results of Fakruddin *et al*. (2012) who found that 47 strains of *BT* fermented glucose, maltose and trehalose whereas they could not grow in the presence of arabinose, mannitol, rhamnose, sorbitol, lactose, or xylose. All strains could grow at low concentration of NaCl (1%) which confirmed with Venosa and Zhu (2003) who reported that the rates of bacterial growth decreased with increasing salinity concentrations.

Biolog GEN III indicated that all bacterial strains belong to *BT*. The GEN III software provided clear indications of the degree of uncertainty for genus and species level identifications. Although the weighted scoring system that used in this study emphasized the importance of identification to species level. The end result of Biolog GEN III is a pattern of coloured wells on the micro plate that is characteristic of that bacterial species. Biolog GEN III applied successfully to a number of taxa such as *Paenibacillus azotofixans* (Pires and Seldin, 1997), *Xanthomonas campestris* pv. *campestris* (Massomo *et al*., 2003) and *Glycine* spp. (Hung and Annapurna, 2004).

The Biolog GEN III system is better at identifying fermentative organisms than non-fermenters. However, it should be noted that biochemically active non-fermenters achieve high identification rates (88%) in the Biolog GEN III system, so a different product may be more suitable for inactive non-fermenters. Biolog GEN III tests a microorganism's ability to utilize or oxidase a panel of carbon sources and this method is used when characterizing bacterial samples within a fixed degree of similarities (Tshikhudo *et al*., 2013).

Giorno *et al*. (2007) stated that *BT* spores cylindrical and ellipsoid in shape while Zolock *et al*. (2006) demonstrated that the spores generally ellipsoidal, 1.5 – 2 μm long and 750 nm –1 μm wide.

Hydrophobicity of the spores, which depends on the presence of the exosporium and appendages, is one of their important physicochemical characteristics. Zolock *et al*. (2006) stated that the investigated spores of *Bacillus cereus* and *BT* have some morphological characteristics, an external sheath (exosporium) and appendages determining their hydrophobicity and capacity for adhesion. Spore appendages involved in adhesion are functionally similar to the fimbria of Gram negative bacteria, such as rhizobacteria (Smirnova *et al*., 2013).

Various rhizobacteria participate in plant colonization, the process involving their surface structures, including fimbria (Berg *et al*., 2005). Spore appendages of bacilli may participate in the interaction with plants. The rhizosphere is a reservoir of microorganisms interacting both with plants and with other hosts. Due to their spore structures, soil bacteria *B. cereus* and *B. thuringiensis* are able to adapt to new conditions and to grow in the intestine of insects and mammals (Stenfors *et al*., 2008).

The scanning electron microscope show surface views of spores at high magnifications can be obtained. Because sample preparation is so simple and rapid, routine examination of spore surface structure for taxonomic purposes could be easily carried out.

REFRENCES

ALAM, K. A., S. A. KHAN, K. SAHELI, M. N. HUDA, M. A. WADUD, M. S. H. REZA, M. E. ALI, C. MANDAL, AND M. A. SALAM. 2008. Mosquitocidal activity of Bti producing cry protein against *Aedes aegypti* mosquito. Research Journal of Environmental Sciences **2**(1): 46 - 51.

ALIGIANNIS, N., E. KALPOUTZAKIS, S. MITAKU, AND B. I. CHINOU. 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agricultural and Food Chemistry **49**: 4168–4170.

BERG, G., L. EBERL, AND A. HARTMANN. 2005. The rhizosphere as reservoir for opportunistic human pathogenic bacteria. Environmental Microbiology **7**: 1673–1685.

BOCHNER, B. R. 2008. Global phenotypic characterization of bacteria. FEMS Microbiology Reviewer **33**: 191–205.

BODADE, R. G., C. N. KHOBRAGADE, P. S. BORKAR, AND R. N. MANWATKAR. 2009. Haemolytic Activity of Some Pathogenic Bacteria in Mammals. Journal of Cell and Tissue Research **9**(2): 1865 - 1868.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). 2006. Performance standards for Antimicrobial Susceptibility Testing, **16th** Informational Supplement.

DA SILVA. N., A. M. G. THULER, I. L. DE ABREU, C. C. DAVOLOS, R. POLANCZYK, AND M.V.F. LEMOS. 2010. Characterization and selection of *Bacillus thuringiensis* isolates effective against *Sitophilus oryzae*. Scientia Agricola **67**(4): 472-478.

FAKRUDDIN, M. D., N. SARKER, M. M. AHMED, AND R. R. NOOR. 2012. Protein Profiling of *Bacillus thuringiensis* Isolated from Agro-forest Soil in Bangladesh. Journal of molecular biology and biotechnology **20** (4): 139-145.

GIORNO, R., J. BOZUE, C. COTE, T. WENZEL, K. S. MOODY, M. MALLOZZI, M. RYAN, R. WANG, R. ZIELKE, J. R. MADDOCK, A.

FRIEDLANDER, S. WELKOS, AND A. DRIKS. 2007. Morphogenesis of the *Bacillus anthracis* spore. Journal of Bacteriology **189**(3):691-705.

HALIMA, H. S., T. HUANG, B. A. ALI, AND Q. XIE. 2006. Differentiation of *Bacillus thuringiensis* and *Escherichia coli* by the randomly amplified polymorphic DNA analysis. Journal of Applied Sciences **6**(7): 1540 - 1546.

HUNG, P. Q., AND K. ANNAPURNA. 2004. Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). Omonrice, **12**:92-101.

JÁNOSI, K., I. HAJTÓS, L. MAKRAI, M. GYURANECZ, J. VARGA, AND L. FODOR. 2009. First isolation of *Histophilus somni* from goats. Veterinary Microbiology **133**: 383–386.

LANKA, S., L. B. BORST, S. K. PATTERSON, AND C. W. MADDOX. 2010. A multiphasic typing approach to subtype *Streptococcus equi* subspecies *equi*. Journal of Veterinary Diagnostic Investigation **22**: 928–936.

MACFADDIN, J. F. 2000. Page 412 in Biochemical Tests for Identification of Medical Bacteria, 2nd Ed. Lippincott Williams & Wilkins, Philadelphia, PA.

MASSOMO, S. M. S., H. NIELSEN, R. B. MABAGALA, K. MANSFELD-GIESE, J. HOCKENHULL, AND C. N. MORTENSEN. **2003**. Identification and characterisation of *Xanthomonas campestris* pv. *campestris* strains from Tanzania by pathogenicity tests, Biolog, rep-PCR and fatty acid methyl ester analysis. European Journal of Plant Pathology **109**:775-789.

PIRES, M. N., AND L. SELDIN. 1997. Evaluation of Biolog system for identification of strains of *Paenibacillus azotofixans*. Antonie Leeuwenhoek **71**:195-200.

SMIBERT, R. M., AND R. K. NOEL. 1994. Methods for General and Molecular Bacteriology, edited by Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, American Society for Microbiology, Washington, DC.

SMIRNOVA, T. A., M. V. ZUBASHEVA, N. V. SHEVLYAGINA, M. A. NIKOLAENKO, AND R. R. AZIZBEKYAN. 2013. Electron Microscopy of the Surfaces of *Bacillus* Spores. Microbiology, 82(6):701–708.

STADTLÄNDER, C. T. K. H. 2007. Scanning Electron Microscopy and Transmission Electron Microscopy of Mollicutes: Challenges and Opportunities. Modern Research and Educational Topics in Microscopy. A Méndez-Vilas and Júnior Díaz (Eds.) 122-131.

STENFORS, A. L. P., A. FAGERLUND, AND P. E. GRANUM. **2008**. From soil to gut: *Bacillus cereus* and poisoning toxins. FEMS Microbiology. Reviewer **32**: 579–606.

TSHIKHUDO, P., R. NNZERU, K. NTUSHELO, AND F. MUDAU, **2013**. Bacterial species identification getting easier. African Journal of Biotechnology **12** (41):5975-5982.

VENOSA, A. D., AND X. ZHU. 2003. Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. Spill Science & Technology Bulletin **8**: 163.

WRAGG, P., L. RANDALL, AND A. M. WHATMORE. 2014. Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flightmass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. Journal of Microbiological Methods **105**: 16–21.

ZOLOCK, R. A., L. GUANGMING, C. BLECKMANN, L. BURGGRAF, AND C. DOUGLAS 2006. Fuller Atomic force microscopy of *Bacillus* spore surface morphology. Micron **37**: 363–369.

التعرف علي بكتيريا باسيلس ثيرونجنسس المعزولة من مصادر مختلفة بإستخدام جهازى Biolog GEN III والمجهر الإلكتروني

هند عبد الحميد محمد حميدو
كلية العلوم – جامعة العريش – العريش - مصر

الملخص العربي

الهدف من هذه الدراسة عزل وتعريف سلالات لبكتيريا باسيلس ثيرونجنسس من مصادر مختلفة والتعرف علي نشاطها البيوكيميائي بإستخدام جهازال Biology GEN III واشكال الجراثيم بإستخدام الميكروسكوب الإلكتروني. وأكدت الدراسة علي وجود خمس عزلات الأكثر نمواً ونشاطاً من حيث مقاومتها لكلوريد الصوديوم عند تركيز ٨٪ ولها قدرة علي التحلل الدموي. كما وجد أنها غير قادرة على البقاء في حرارة ٥٠ درجة مئوية. أظهرت العزلات نشاطاً إيجابياً لإختبار الكاتليز، وإختبارأحمرالميثيلين، واختبار arginin decarboxylation و citrate utilization وأعطت العزلات نشاطا سلبياً لاختبارات الأوكسيديز والإندول.

اثبتت الدراسة قدرة هذه الكائنات علي تخمر الجلوكوز، والسكروز، المالتوز والفركتوز، الرافينوز، النشا، الجلسرين، البكتين والجيلاتين أيضاً في حين أنها لا تخمر المانيتول، الأرابيتول، الرامنوز، اللاكتوز، المانوز أو السوربيتول. وكانت جميع العزلات إيجابية لصبغة جرام وحساسة للبنسلين، توبراميسين، البكترسين والأموكسيسيلين / حامض الكلفولنيك بينما كانت مقاومة للكليندامايسين، نيتروفورانتوين، الكانديسيدين والكلارمفنيكول. تم مسح وتصوير جراثيم بكتريا الباسيلس ثيرونجنسس بواسطة المجهر الإلكتروني الماسح. وقد وصفت الجراثيم بأنها أسطوانية الشكل، سطحها أملس وفي بعض الأحيان مضلع قليلاً جداً وكانت مغطاة بـExosporium.