Natural Associations between Symbionts *Photorhabdus Spp.* and *Xenorhabdus spp.* and Bacteria Related to *Ochrobactrum anthropi, Bacillus pumilus and Enterobacter cloacae*

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Abstract: *Xenorhabdus spp.* and *Photorhabdus spp.* are symbiotic bacteria that produce numerous metabolites of insecticidal and bactericidal impact. It was thought that these biological metabolites gave them the privilege of predominance in the gut of entomopathogenic nematodes (EPNs). The aim of this work was to investigate and characterize the associated bacteria with symbiotic bacteria *Photorhabdus* and *Xenorhabdus in-vitro* culture of EPNs (*Heterorhabditis indica* RM1, *Heterorhabditis sp.* S1 and *Steinernema abbasi*). The gut flora of EPNs were isolated and characterized morphologically and biochemically. The isolated associated strains were completely identified by restriction fragment length polymorphism and sequence analyses of PCR-amplified 16S rRNA. The isolated strains were *Ochrobactrum anthropi* and *Bacillus pumilus* associated with *Photorhabdus luminescens akhurstii* in the gut of *H. indica* RM1 and *Heterorhabditis sp.* S1 (Egyptian isolates). Also *Enterobacter cloacae* were associated with *X. indica* in the gut of *S. abbasi* (Omani isolate). These associated isolates are raising the concern about their effect in epidemiological maps in Egypt, Oman and their significance as public health threats. Regarding the common use of (EPNs), previous clinical case reports for these associated isolates and their developing record in human infectivity we should pronounce alarm of possible biological hazard of intensive use of EPNs without monitoring the hidden associated bacteria during their manufacture.

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1. Introduction

Entomopathogenic nematodes (EPNs) are biological control agents that are pathogenic to a wide range of insect pests **(*Grewal et al.*, 2005)**. Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are known to be symbiotically associated with the soil dwelling EPNs of the family *Steinernematidae* and *Heterorhabditidae*, respectively **(*Marokhazi et al.*, 2004)**.

It has been assumed that the monoxenic association between the nematode and its symbiont is referred to bactericidal compounds produced by the symbiont during the reproduction of the nematode in the insect. All *Steinernema* spp. and *Heterorhabditis* spp. carry in their gut symbiotic bacteria of the genus *Xenorhabdus* **(*Thomas* and *Poinar,* 1979*; Akhurst* and *Boemare, 1988*)** and genus *Photorhabdus* **(*Boemare et al.*, 1993; *Akhurst et al.*, 1996; *Fischer-Le Saux et al.*, 1999) respectively**. These bactericidal compounds are believed to avert the development of other bacteria in the insect cadaver. However, several reports have shown that there are infrequently bacteria other than the unique symbiotic bacteria in the gut of nematodes. ***Lysenko*** and ***Weise*** **(1974)** isolated bacteria such as *Alcaligenes*, *Pseudomonas* and *Acinetobacter spp.* from *Steinernema carpocapsae*. Also ***Boemare*** **(1983)** isolated other associated bacteria such as *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans* and *Serratia liquefaciens* from *S. carpocapsae*. Similar observations were reported for *Steinernema scapterisci*, which was transferred from South America and sub cultured many times in Florida. This nematode was associated with *Ochrobactrum anthropi*, *Paracoccus denitricans*, *Pseudomonas maltophilia* and *Xenorhabdus spp.* **(*Aguillera* and *Smart,* 1993; *Aguillera et al.*, 1993).** It is noteworthy to mention that ***Babic et al.*** **(2000)** studied the naturally occurring bacteria isolated in association with *Photorhabdus luminescens* from tropical *Heterorhabditis spp.* in the Caribbean basin by using conventional phenotypic tests, restriction fragment length polymorphism and sequence analyses of PCR - amplified 16S rRNA genes (16S rDNAs) and they recorded the isolation of bacteria characterized as *Ochrobactrum* spp. and associated with the natural symbiont *Photorhabdus luminescens* subsp. *akhurstii* in the entomopathogenic nematode *Hterorhabditis indica* from the Caribbean basin. Some of these associated bacteria are of great benefit and in complete harmony with *Xenorhabdus, Photorhabdus* spp. bacteria as many researchers

showed a great similarity in many biological metabolites as proteases (zinc metaloproteases), this similarity is not restricted only to chemical nature (***Bowen et al.,* 2003**) but also extended to include the genes coding for such enzyme as many scientists proved that there is many sharing genes coding for enzymes responsible for hemolysis of sheep RBCs (hemolysis similarity between *P. luminescens* and *Serratia marcescens*) (***Brillard et al*., 2002**).

Actually the similarity between *Xenorhabdus-Photorhabdus* with associated bacteria not restricted only to similarity in bacterial enzymes it also extended to the similarity of bacterial toxins **(*Hertle,* 2005**) which plays a significant role in the control and destruction of insect host and their toxicity through secretion of these toxins in insect hemolymph as what occurs with *Serratia marcescens* which plays main role in inducing of infectivity in *Galleria mellonella* larvae by *S. carpocapsae filipjev* (***Ortega-Estrada et al.,* 2012).** This phenomena was also reported by ***Abebe et al.* (2011)** where *Serratia sp*. was able to kill *G. mellonella* in a manner similar to that induced by *Heterorhabditis* and *Photorhabdus* species. ***Sundra et al.* (1993**) postulated that *X. nematophila* indole antibiotics act as regulator to one of the associated bacteria (*P. putida*) and the upper hand seems to be for *Xenorhabdus* bacteria.

Bacillus spp. N. strain associated with *Rhabditidis* (EPNs) bacteria produces bioactive compounds (antibacterial and fungicidal) **(*Kumar et al.,* 2012b)** and these associated bacteria was isolated from 3rd stage infected juveniles of the nematode sample collected from hemolymph of *Galleria mellonella* (***Kumar et al.,* 2012a**). Also *Serratia sp.* showed a similar action against *G. mellonella* (***Abebe et al., 2011***).

The harmony between *Xenorhabdus, Photorhabdus* spp. and associated bacteria not only restricted to production of similar enzymes or carrying the same coding genes or even in synergistic mode of action for controlling infectivity of insects but many scientists extended their work to prove the evolutionary relationship between *Xenorhabdus, Photorhabdus spp.* and their associated bacteria where ***Moran et al.* (2005)** proved the evolutionary relationship between *Serratia spp.* and *Photorhabdus* spp. by phylogenic analysis of partial sequences of genes by 16S rRNA gene sequence.

The apprehension issue here is the emphasis of the biohazard activity of some of these associated bacteria especially that some strains like *E. faecalis* produced cytolysin toxin that causes urinary tract infection, bacteremia and endocarditis (***Fieldhouse et al.,* 2010)** like what reported for *Photorhabdus lumensense* and *Photorhabdus asymbiotica* in North America and Australia by ***Peel et al. (1999)***.

It must be kept in mind that *Xenorhabdus* and *Photorhabdus* are used intensely in commercial products for biological control of agricultural insects. So, great consideration must be given for the bacterial load carried by entomopathogenic nematodes *in-vitro* culture. This research targeted exploration of bacteria that present in association with *Photorhabdus luminescens akhurstii* isolated from EPNs *Heterorhabditis indica* RM1 and *Heterorhaditis* sp. S1 isolated from Egyptian soil Also *Xenorhabdus indica* isolated from the Omani EPNs *S. abbasi*.

2. Materials and Methods

Nematode propagation

Heterorhabditis indica RM1 and *Heterorhaditis* sp. S1 are entomopathogenic nematodes (EPNs) isolated from Egyptian soil while *Steinernema abbasi* Ab nematode was isolated from Omani soil. They were maintained in the laboratory by several passages through last-instars larvae of *Galleria mellonella* according to ***Dutky et al.* (1964)** in the Department of Parasitology and Animal Diseases, National Research Centre, Dokki, Egypt.

Bacterial isolates

The origins of the bacterial strains were listed in table 1. *Xenorhabdus nematophila* 19061, and *Photorhabdus luminescens* TT01 as standard strains were obtained from University of Wisconsin, Milwaukee. The bacteria associated with infective juveniles were isolated by the hanging-drop technique **(*Poinar* and *Thomas,* 1966)**. A sterile drop of insect hemolymph was collected after surface sterilization of infected *Galleria mellonella* (10 min in 1% sodium hypochlorite solution) and rinsed three times in sterile water. The microorganisms developing in the drop after 48 h at 26 °C were streaked on MacConkey agar. Unknown bacteria associated with *Photorhabdus luminescens akhurstii* RM1, S1 and *Xenorhabdus indica* Ab phase I, which was found in reared *Heterorhabditis* and *Steinernema* nematodes were repeatedly isolated on this medium. These *Photorhabdus luminescens* - associated strains were designated as SIA and RMIA while those associated with *Xenorhabdus indica* were designated as AbA.

Culture conditions

The optimal growth temperature was determined by streaking each isolate on NBTA medium (nutrient agar supplemented with 25 mg bromothymol blue l^{-1} and 40 mg triphenyltetrazolium chloride l^{-1}) and incubating at 26 °C. All the *Photorhabdus* and *Xenorhabdus*-associated isolates grew better at 26 °C, and consequently all the biochemical tests described below were conducted at this temperature. Isolates were grown in Luria broth (LB) (Difco- Fisher Scientific) on a shaking rack for 24 h. The *Photorhabdus* and *Xenorhabdus*- associated isolates were maintained on nutrient agar plates at

5 °C. *Xenorhabdus nematophila* 19061 and *Photorhabdus luminescens* TT01 strains are usually cultivated at 26 °C. They were maintained on NBTA medium at 15°C **(*Akhurst,* 1980)**.

Phenotypic characterization of the *Photorhabdus* and *Xenorhabdus*- associated isolates.

Phenotypic characterization was conducted according to ***Bergey's Manual* (*Holt et al.,* 1994)**, and later reports **(*Alnor et al.,* 1994** and ***Velasco et al.,* 1998)** and additional tests summarized below. Colonial morphology was observed on nutrient agar and the diameter of colonies was measured after 24 and 48h in five independent experiments. Dye adsorption on MacConkey agar or NBTA, and the test for bioluminescence were conducted according to ***Boemare*** and ***Akhurst* (1988)**. The cell wall was characterized by the Gram stain test **(*Cerny,* 1976)**. The biochemical reactions were carried out according to ***Holt et al.,* 1994** and they included the following: catalase reaction, oxidase reaction, sugar fermentation (lactose, sucrose, esculine), urease reaction, growth on bile agar, nitrate reduction test, indol reaction, MRVP reaction. Haemolysis was determined on Tryptic Soy Agar (BioMerieux) supplemented with 10 % (v/v) sterile defibrinated sheep blood (BioMerieux).

DNA extraction and PCR amplification of 16S rRNA genes

DNA extraction and PCR amplification of 16S rRNA genes was carried out according to ***Van der Hoeven et al.* (2008)**. Based on morphological and Gram stain results, bacterial isolates were chosen for amplification of 16S rRNA genes. In most cases, PCR was carried out using a direct colony PCR approach. In cases where a product was not obtained, DNA was extracted from 2-mL LB overnight cultures of individual isolates grown at 26 °C using the Edge BioSystems Bacterial Genomic DNA Purification Kit. The universal bacterial primers 50-GTTTGATCCTGGCTCAG-30 (11F) and 5´-ACGGYTACCTTGTTACGACTT-3´ (1512R) were used in the PCR reaction. A small amount of cells was scraped from the colony using a sterile pipette tip, resuspended in 3 mL distilled PCR-grade water and boiled for 5 min. The boiled cells were added to 47 µl of a PCR mixture containing 2 mM forward and reverse primers, 2.5 mM $MgCl_2$, 200 mM dNTPs, 5µl $MgCl_2$-free 10X Taq polymerase buffer and 1.25 U Taq DNA polymerase (Promega Co.). PCR amplification was carried out as follows: 30 s denaturing at 94 °C, 30 s annealing at 55 °C and 1-min extension at 72 °C for 30 cycles. PCR products were purified using the High Pure PCR Product Purification Kit (Roche) and analyzed on 0.7% agarose gels (Ref.??).

Nucleotide sequence analysis of purified PCR products

Nucleotide sequence analysis of purified PCR products was performed at the University of Chicago, Cancer Research Center, DNA Sequencing and Genotyping Facility. BLASTN search of the NCBI database was used for genus designation with sequences of length c. 760 bp. To further confirm identification and grouping of genera, sequences were aligned with CLUSTALW and compared with the sequences in Ribosomal Database Project II (RDP-II), using the Sequence Match function (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). In all cases, 16S rRNA gene analysis was consistent with microbiological data.

3. Results

The isolated strains from *Heterorhabditis indica* RM1 and *Heterorhabditis* spp. S1 which are usually engaged with *Photorhabdus luminescens akhurstii* as a symbiotic bacteria was unexpectedly associated with another Gram negative small bacilli designated as SIA and RMIA (Table 1) which gave red coloration and different biochemical reaction as illustrated in table 2.

Also, another bacterial strain was isolated from *Steinernema abbasi* Ab which is usually occupied with *Xenorhabdus indica*. This Gram negative small bacillus was designated as AbA (Table 1). It gave red color colony on NBTA agar and different biochemical reaction as illustrated in table 2.

Table 1: *Photorhabdus* and *Xenorhabdus* - associated isolates and laboratory strains used in this study

Insect Host	Entomopathogenic nematodes (EPNs)	Symbiotic bacteria	Associated isolates
Galleria melonella	*Heterorhabditis indica* RM1	*Photorhabdus luminescens akhurstii*	**RMIA**
	Heterorhabditis sp. S1		**SIA**
	Steinernema abbasi Ab	*Xenorhabdus indica*	**AbA**
	Steinernema carpocapsi	*Xenorhabdus nematophila* 19061	**Standard laboratory strains from University of Wisconsin, Milwaukee, USA**
	Heterorhabditis indica	*Photorhabdus luminescens* TT01	

The biochemical reactions of strain SIA were summarized in table 2 and the most prominent results were recapitulated in the lacking of luminous character of genus *Photorhabdus*, weak positive catalase reaction, incapability of sugar fermentation (sucrose, lactose and esculin), positive urease reaction and loss of capability to grow on bile agar.

The biochemical reactions of strain RMIA as summarized in table 2 showed remarkable lacking of luminous character of genus *Photorhabdus*, positive

catalase reaction, positive sugar fermentation (sucrose, lactose and esculin), positive VP reaction and had the capability to grow on bile agar (table 2).

Table 2: Phenotypic characters distinguishing *Photorhabdus* and *Xenorhabdus*-associated isolates.

Phenotypic character	*P. luminescens TT01*	*X. nematophila 19061*	*AbA*	*SIA*	*RMIA*
Colony color on NBTA agar	Blue	Blue	Red	Red	Red
Luminescent	[+ve]	-ve	-ve	-ve	-ve
Gram's stain	-ve	-ve	-ve	-ve	-ve
Shape	Rods	Rods	Rods	Rods	Rods
Motility	Motile	Motile	Motile	Motile	Motile
Catalase reaction	-ve	-ve	[+ve]	[+ve] W	[+ve]
Oxidase reaction	-ve	-ve	-ve	[+ve] W	-ve
Sugar fermentation:					
lactose	[+ve] W	[+ve] W	[+ve] W	-ve	[+ve]
Sucrose	[+ve] W	[+ve] W	[+ve] S	-ve	[+ve] S
Esculin	[+ve] W	-ve	-ve	-ve	[+ve] S
Urease reaction	-ve	-ve	-ve	[+ve]	-ve
Growth on bile agar	[+ve]	-ve	-ve	-ve	[+ve]
Nitrate reduction test	-ve	-ve	[+ve]	-ve	-ve
Indole reaction	-ve	-ve	-ve	-ve	-ve
MR reaction.	-ve	-ve	-ve	-ve	-ve
VP reaction	-ve	-ve	[+ve] ^{W}	-ve	[+ve] ^{S}
Hemolysis	-ve	[+ve]	-ve	-ve	-ve

All tests were done at 37°C, weak positive [+ve] ^{W}, strong positive, [+ve] ^{S}, positive [+ve], negative -ve. The most discriminatory characters are shown in bold.

The biochemical reactions of AbA were characterized by positive catalase reaction, positive nitrate reduction test, and positive VP reaction and the rest of the reactions were similar to *X. nematophila 19061* except colony color and hemolysis characters as shown in table 2.

Bacterial identification by 16SrRNA

The identification of the isolated strains were confirmed by using specific PCR probe using specific primers where the bacterial PCR products (1.4 kbp) were screened using 0.7 agarose gel electrophoresis (Fig. 1).

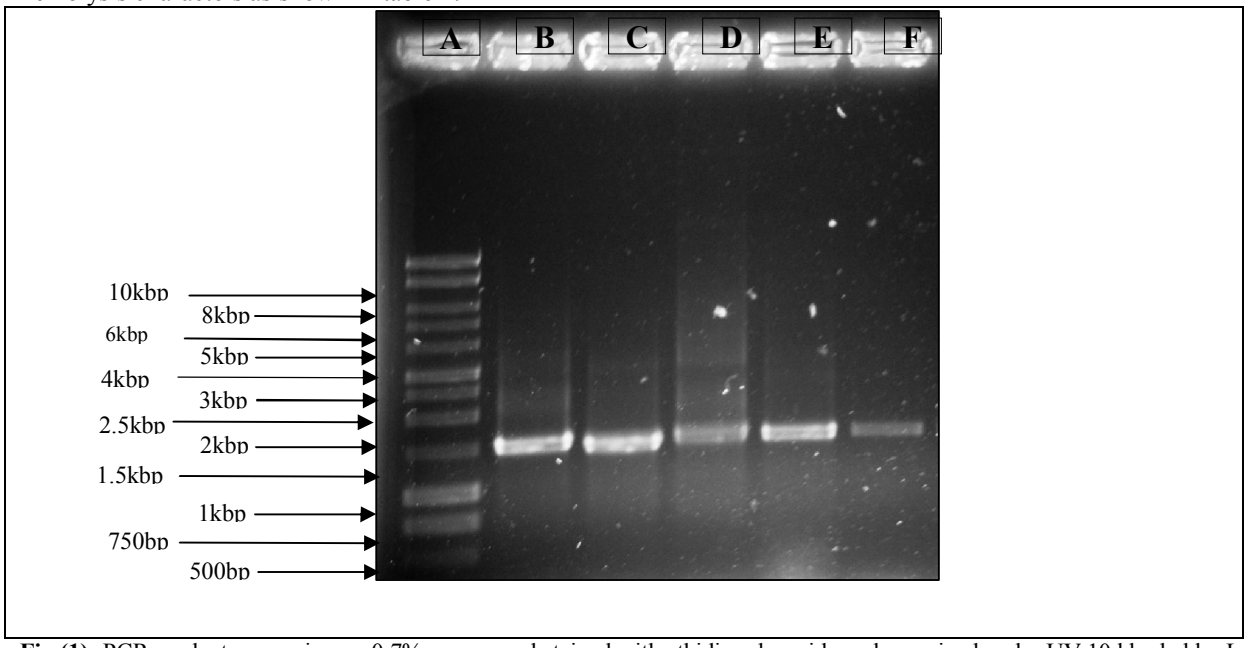

Fig (1): PCR products screening on 0.7% agarose gel stained with ethidium bromide and examined under UV 10 kbp ladder Lane (A), *P. luminescens* (TT01) Lane (B), *X. nematophila* (19061) lane (C), *AbA* isolate Lane (D), *SIA* isolate Lane (E) and *RMIA* isolate Lane (F).

Nucleotide sequence analysis of purified PCR products.

The Nucleotide sequence analysis of purified PCR products (Done at Chicago Cancer Research Center) and the BLASTN search of the NCBI database resulted in identification of isolate SIA as *Ochrobactrum anthropi*, isolate RMIA was defined as *Bacillus pumilus,* while isolate AbA was defined as *Enterobacter cloacae*.

For further confirm identification and grouping of genera, sequences were aligned with CLUSTALW and compared with the sequences in Ribosomal Database Project II (RDP-II), using the Sequence Match function (http://rdp.cme.msu.edu/seqmatch/ seqmatch_intro.jsp).

Table 3: Nucleotide sequence analysis of purified PCR products

Designated Code	Defined strain	Max score	Total score	Query coverage %	Error value	Max ident. %
SIA	*Ochrobactrum anthropi* strain SRK5	**1881**	1881	100	0.0	99
RMIA	*Bacillus pumilus* strain CTSP14	**1482**	1482	100	0.0	99
AbA	*Enterobacter cloacae* isolate	**1796**	1796	100	0.0	100

4. Discussion

The present work reported the isolation of three strains according to 16sRNA plast. These strains are *Ochrobactrum anthropi*, *Bacillus pumilus* and *Enterobacter cloacae*. These strains were associated with the symbiotic bacteria as they were isolated from the infected insect (*Galleria mellonella*) hemolymph. *Ochrobactrum anthropi* and *Bacillus pumilus* were associated with *Photorhabdus luminescens akhurstii* while *E. cloacae* was associated with *Xenorhabdus indica*.

The sterilization technique followed during the process of hemolymph collection from infected insect is well established by many authors in this field (***Razia et al., 2011***) and the frequency of isolation gave no doubt in any possibility of contamination.

Some of the isolated associated strains like *O. anthropi* had been recognized and mentioned in previous reports (***Razia et al.,* 2011**) while according to our knowledge *B. pumilus* and *E. cloacae* was not reported previously in association with symbiotic bacteria. The main concern here is the health hazard significance of these associated bacteria with the symbiotic bacteria (*Xenorhabdus* and *Photorhabdus* sp.) **(*Babic et al.,* 2000)**. The contribution of symbiotic bacteria in *in-vitro* culture of entomopathogenic nematodes as biological pesticides is well established and well spread all over the world. It was believed that the antimicrobial toxins and metabolites of symbiotic bacteria gave no chance for growth of any other microorganisms but these recent reports smashed out this theory and raised an urgent query about the pathogenicity and the infectivity of such associated bacteria. In order to estimate the bio safety of these associated bacteria we should appraise the nature of isolated strains and the previous clinical case reports interrelated with them.

Ochrobactrum anthropi is a Gram-negative, aerobic bacillus previously classified by Center of Disease Control and prevention (CDC) as group Vd. *O. anthropi* is motile organism characterized by production of urease and oxidase (***Holmes et al.,* 1988; *Bruckner*** and ***Colonna.* 1993),** it is wide spread ecologically in nature including soil and water and contaminated pharmaceuticals (***Barson et al.,* 1987**; ***Holmes et al.,* 1988**; ***Deliere et al.,*** 2000; ***Peltroche-Llacsahuanga et al.,* 2000)**. *Ochrobactrum anthropi* had been isolated from different human clinical sources as blood, urine, wounds, feces and oral and vaginal secretions, leading to its specific epithet anthropi **(*Holmes et al.,* 1988)**.

Ochrobactrum anthropi is believed to be an opportunistic organism that can affect mainly immune compromised individuals and indwelling catheters **(*Vaidya et al.,* 2006)** and the first record of clinical infection with *O. anthropi* was recorded at 1980 in debilitated patients with pancreatic abscesses **(*Appelbaum and Campbell* 1980).** However lately many clinical cases reported affections with *O. anthropi* in healthy individuals which confirm their ability to cause infection **(*Mahmood, et al.,* 2000*; Vaidya et al.,* 2006; *Alparslan et al.,* 2012; *Quintela Obregón et al.,* 2012).** There are many evident reports supporting this finding as *O. anthropi* caused endophthalmitis **(*Chiang et al.,* 2009** and ***Song et al.,* 2007)**, osteomyelitis **(*Wheen et al.,* 2002)**, meningitis **(*Christenson et al.,* 1997)**, sepsis associated with an infected venous catheter **(*Wi and Peck* 2010)**, contaminated pharmaceuticals **(*Kettaneh, et al.,* 2003**. and ***Mahmood, et al.,* 2000)**, pelvic abscess **(*Vaidya et al.,* 2006)**, septic shock **(*Ozdemir et al.,* 2006)**, and peritonitis (***Alparslan et al.,* 2012**). So we can conclude that *O. anthropi* can affect healthy individuals as well as immune compromised patients. The other public health issue that we must pointed to is the developed resistance of *O. anthropi* to antibiotics such as beta-lactams, ampicillin-clavulanate, pipracellin-tazobactams, cefotaxime, ceftriaxone, and aztreonam **(*Cieslak et al.,* 1996)**.

Ochrobactrum anthropi has been isolated from different geographical regions like Caribbean islands (***Babic et al.,* 2000)** and France (***Romano et al.,* 2009**)

and many other places in the world and here in Egypt we reported the isolation of *O. anthropi* in association with the symbiotic bacteria *Photorhabdus luminescence akhurstii* from the native isolates of *Heterorhabditis indica* RM1.

The second isolated strain was *Bacillus pumilus* which gave the identical phenotypic characters of the known strain as mentioned by ***O'Donnell et al.* (1980)**. It is also, noteworthy to highlight the significance of the other associated bacteria isolated in this study like *B. pumilus*. Other reports mentioned that *Bacillus* N strain is isolated from novel *Rhabditid* entomopathogenic nematode in India and this strain shows antibacterial and fungicidal activity (***Kumar et al*., 2012 a & b)** so this associated bacteria may had a significant synergistic effect in controlling the biological environment in the gut of EPNs and their host insect.

Enterobacter cloacae was isolated as an associated organism with *Xenorhabdus indica* which reside from *Galleria mellonella* infected with *Stienernema abassi* Ab. The isolated strain showed identical phenotypic characters to identical strain of *E. cloacae* recorded by known authors like ***Wang et al.* (1989)**. *Enterobacter cloacae* is widely dispersed in nature. However it is less frequently reported as endemic infectious agent for human but outbreaks were reported due to lack of hygienic measures and sanitation **(*Eckmanns et al.*, 2000).** *Enterobacter cloacae* as a significant nosocomial infective agent was reported in water and even in newly born intensive care unit (CIU) **(*LaTuga et al.*, 2011; *Crémet et al.*, 2012)**. Also, *E. cloacae* recorded developed resistance to many antibiotics which gave threaten alarm for public health concern **(*Mezzatesta et al.*, 2012)**.

It can be conclude that our results are in agreement with ***Babic et al.*, 2000** and conflict with what claimed by ***Boemare et al.* (1996),** that monoxenic nematodes obtained from the combination of surface axenized eggs with the natural symbiont should be used for this purpose.

So this work recommends that the *in-vitro* culture of entomopathogenic nematodes for mass production purposes should be strictly controlled to prevent any contamination and to avoid epidemiological spreading of the pathogenic associated bacteria.

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