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Genetic Characterization of *Corynebacterium pseudotuberculosis* Isolates in Egypt

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ABSTRACT

Corynebacterium pseudotuberculosis is a small Gram-positive bacillus containing mycolic acid in the structure of the cell wall. The bacterium is responsible for Caseous Lymphadenitis (CLA) in small ruminants (sheep and goats). The bacteria are also responsible for Ulcerative Lymphangitis in equines. The disease causes great economic loss in the animal industry. This work aimed to check the ability of Quadruplex PCR (Q-PCR) for genotyping and identification of Egyptian isolates of *C. pseudotuberculosis* and sequence analysis of phospholipase D (*PLD)* gene of local isolates. Four of *C. pseudotuberculosis* local isolates previously biochemically identified were tested for *narG* gene (nitrate reductase gene). Both nitrate negative biovar (ovis) and nitrate positive biovar (equi) showed a positive result for *16S rRNA, rpoB* and *PLD* genes of *C. pseudotuberculosis* species. The sequence analysis of our local isolates PLD gene revealed minor changes in *PLD* proteins between ovine and equine strains compared with other published *PLD* sequences in GenBank. It was concluded that the Q-PCR method is able to differentiate between *C. pseudotuberculosis equi* and *ovis* biovars. Also, the sequence of *PLD* gene of local isolates representing the two biovars revealed some variation, which leads to an accurate diagnosis of *C. pseudotuberculosis* biovars and generates a mapping of immerged local isolates. Further, the PCR and sequence of these isolates provide rapid and accurate genotyping, especially with hyperimmune serum unavailability.

Keywords: *C. pseudotuberculosis*, *PLD* gene, Quadruplex PCR, Sequence analysis.

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INTRODUCTION

Corynebacterium pseudotuberculosis is a Gram-positive and small bacillus. It is the causative agent of Caseous Lymphadenitis (CLA) in goats and sheep, while in buffaloes and cattle, it causes a disease known as Oedematose Skin Disease (OSD), but in equine it causes abscesses and ulcerative lymphangitis. It was also recorded in other hosts, including camels and rare in humans **(Tejedor *et al.*, 2008)**. The classification of *C. pseudotuberculosis* into two biovars depended on the bacteria's ability to convert nitrate to nitrite. Biovar equi strains are nitrate positive and infect both horses and buffalos, while biovar ovis are known to be nitrate negative and it is the causative agent for CLA in goats and sheep **(Biberstein *et al.*, 1971; Selim., 2001; Baird and Fontaine,2007)**. The two biovars could be isolated from cattle, while the tissue tropism may differ between them, biovar ovis tends to infect the skin and mammary gland **(Yeruham *et al.*,1996 and 1997)**, while biovar equi tend more to cause ulcerative lymphangitis. Low milk production and weight loss are considered major economic losses due to *C. pseudotuberculosis* infection. Also, The difficult eradication of CLA as result of ineffective antimicrobial therapy **(Williamson, 2001)**. Several

detection methods were used for diagnosis of *C. pseudotuberculosis*, both direct and indirect, such as *PLD* antigen-based ELISA, Synergistic Hemolysis Inhibition Test **(Brown *et al.*,1987; Dercksen *et al.*, 2000)**,

Microagglutination Assay, Complement fixation test **(Shigidi, 1979; Menzies and Muckle,1989)** and a multiplex PCR **(Pacheco *et al.*,2007 and Almeida *et al.*,2017(a))**. These tests are used for laboratory identification and could distinguish between *C. pseudotuberculosis* biovars in parallel with the biochemical tests, especially the nitrate reduction test. The diver gene among biovars seems to be related to virulence and could be related to tissue and host specificity too **(Williams and Sadler, 1971 and Sankarasubramanian *et al.*, 2016)** and may show various symptoms in the diseased animals **(Yeruham *et al.*,1996 and 1997; Selim, 2001; Baird and Fontaine, 2007)**. The biovar's determination is crucial to help control the disease and may help to understand its epidemiology. Actually, the standard test used to differentiate *C. pseudotuberculosis* biovars equi and ovis was the nitrate reduction test **(Biberstein *et al.*,1971 and Guimarães *et al.*,2011)**. The existence of nitrate reduction operon with15 genes in only biovar equi including the *narKGHJI* operon strains is considered the main genetic trait that differentiates the biovars of *C. pseudotuberculosis* **(Almeida *et al.*,2017(b))**.

So, PCR assay is most important in the differentiation of two biovars **(Steinman *et al.*, 1999; Yeruham *et al.*, 2003 and Guimarães *et al.*,2011)**. Other effective phenotypic tests for identification of *C. pseudotuberculosis* have high cost or unavailable for some labs, but currently, most microbiology laboratories are changing to modern diagnostic molecular techniques like PCR and sequence analysis rather than biochemical tests **(Raoul *et al.*, 2004; Pacheco *et al.*,2007; Goncalves *et al.*,2014; Oliveira *et al.*,2016 and Almeida *et al.*,2017(a))** which have the advantage of saving time and efforts **(Langoni *et al.*,2017)**. This work aimed to ensure Q-PCR assay accuracy with sequence analysis of *PLD* gene for concurrent identification and genotyping of *C. pseudotuberculosis* local isolates.

MATERIALS AND METHODS

Bacterial strains:

Four lyophilized locally isolated *C. pseudotuberculosis* strains were used. The isolates were obtained from different governorates and hosts within different periods, supplied by Dr. Roukaya M. O. are shown in Table (1). The isolates were identified biochemically **(Khamis *et al.*, 2004 and Roukaya,1982)**.

Table 1: *C. pseudotuberculosis* from different governorates and different hosts during different periods.

Local Strain	Host	Governments	Year of Isolation
CpO/S Egy.VSVRI	Sheep	Zagazeg	1981
CpO/G Egy.VSVRI	Goat	Monofia	1980
Cp/B Egy.VSVRI	Buffalo	Monofia	1980
Cp/C Egy.VSVRI	Cattle	Monofia	1981

DNA extraction and Multiplex PCR

Extraction of DNA occurred by rapid boiling (**Abdelhai *et al.*, 2016).** The primers used in this study were listed in Table (2). Primers used to target *16S rRNA, rpoB, PLD,* and *narG* genes of *C. pseudotuberculosis* **(Cetinkaya *et al.*,2002; Khamis *et al.*,2004; Pacheco *et al.*,2007 and Almeida *et al.*,2017(a))**. Q-PCR was carried out to reach a final volume of 50 µL, including 1µLof each primer, 5 µL of genomic DNA, 25 µL of Green *Taq* DNA Master Mix (Thermo Scientific) and complete the volume with d. d. H2O. The Applied Biosystem, GeneAmp PCR system thermal cycler 9700, USA was used in the process of amplification, as the following steps, the initial denaturation at 95 °C for ten minutes; followed by 30 cycles: 95 °C for the 30s, 58 °C for the 30s and 72 °C for 1.5 min, final extension at 72 °C for 10 min. The products were subjected to electrophoresis in 1.5% (w/v) agarose gel and visualized under Ultra Violet (UV) light by ethidium bromide staining.

Singleplex PCR of whole *PLD* gene of four local *C. pseudotuberculosis* strains for sequencing

PCR of whole *PLD* gene was performed by the specific primer of *PLD* **(Aquino *et al.*,2013)**, *PLD*W/F and *PLD*W/R are listed in Table (2). PCR was performed to reach 50 µL (final volume), having 5 µL of genomic DNA, 1 µL of each primer, 25 µL of Green *Taq* DNA Master Mix and the volume was completed with d. d. H2O. Amplification reaction consisted of the following steps: the first denaturation at 94 °C for ten mints; 40 cycles of denaturation at 94 °C for 40 s, annealing were performed at 58 °C for the 40s, and extension at 72 °C for 40s followed by final extension step at 72 °C for ten mints. PCR products were purified for sequence analysis using QIA quick gel extraction kit (Qiagen, Germany). Sequence reactions were performed at the GATC Company, Germany, using (ABI 3730xI DNA) sequencer . The sequence of the *PLD* gene of four local Egyptian isolates was compared with the other ten *PLD* of *C. pseudotuberculosis* strains published sequences on Gen Bank (table 3) and homology % was calculated by Meg Align (DNA STAR, Laser gene, Version 7, USA).

Table 2: The oligonucleotide primer sequences

Target gene	Primer	Sequence(5´-3´)	Amplicon size(bp)	Reference
16S rRNA	Forward	ACCGCACTTTAGTGTGTGTG	816	**Cetinkaya *et al.*,2002**
	Reverse	TCTCTACGCCGATCTTGTAT		
rpoB	Forward	CGTATGAACATCGGCCAGGT	446	**Khamis *et al.*,2004**
	Reverse	TCCATTTCGCCGAAGCGCTG		
Pld	Forward	ATAAGCGTAAGCAGGGAGCA	203	**Pacheco *et al.*,2007**
	Reverse	ATCAGCGGTGATTGTCTTCCAGG		
narG	Forward	ACCCGTACTTGCACTCTTTC	612	**Almeida *et al.*,2017(a)**
	Reverse	AGTCAGTACTTCCGCAGGTC		
*PLD*W	Forward	ATGAGGGAGAAAGTTGTTTTA	942	**Aquino *et al.*,2013**
	Reverse	TCACCACGGGTTATCCGC		

Table 3: *C. pseudotuberculosis* strains published sequences on GenBank

Accession No.	Identification of C. pseudotuberculosis isolates	Biovar	Host	Country
CP003421.3	*Corynebacterium pseudotuberculosis* 31, complete genome	Equi	Buffalo	Egypt
CP003061.2	*Corynebacterium pseudotuberculosis* CIP 52.97, complete genome	Equi	Horse	Kenya
CP003540.2	*Corynebacterium pseudotuberculosis* 258, complete genome	Equi	Horse	Belgium
CP003082.1	*Corynebacterium pseudotuberculosis* 1/06-A, complete genome	Equi	Horse	USA
L16586.1	*Corynebacterium pseudotuberculosis* phospholipase D(PLD) gene, complete CDs	Equi	-	-
CP021251.1	*Corynebacterium pseudotuberculosis* strain ATCC 19410 chromosome, complete genome	-	Bovine	Brazil
CP002251.2	*Corynebacterium pseudotuberculosis* I19, complete genome	Ovis	Cow	Israel
CP003062.1	*Corynebacterium pseudotuberculosis* 42/02-A, complete genome	Ovis	Sheep	Australia
CP001809.2	*Corynebacterium pseudotuberculosis* 1002, complete genome	Ovis	Goat	Brazil
CP001829.1	*Corynebacterium pseudotuberculosis* C231, complete genome	Ovis	Sheep	Australia

RESULTS

Specificity and assay of the Multiplex PCR

Purified genomic DNAs were used to evaluate the specificity of the multiplex PCR assay. When tested with DNA from bacterial isolates taxonomically related to *C. pseudotuberculosis* biovar ovis from goat and sheep and *C. pseudotuberculosis* biovar equi from buffalo and cattle gave a similar profile of Multiplex PCR, with the 816,446 and 203 bp amplicons corresponding to the *16S rRNA, rpoB* and PLD genes, respectively Fig. (1). **Khamis *et al.*, 2005**, showed that the tested Corynebacterial species had generated an amplicon of ~446 bp, corresponding to the *rpoB* gene. The PLD gene product was found only in *C. pseudotuberculosis* isolates demonstrating the Multiplex PCR assay capability to differentiate this bacterium from other *Corynebacterium* species. The multiplex PCR assay that targets *16S rRNA, rpoB,* and PLD genes was improved by adding *C. pseudotuberculosis* biovar-specific primers for the *narG* gene (Table 2) which was only found in *C. pseudotuberculosis* isolates biovar equi from cattle and buffalo (Fig. 2).

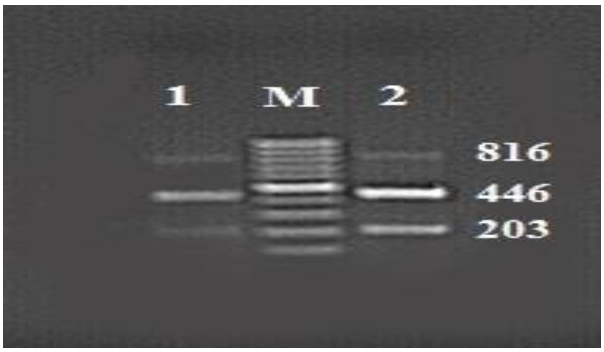

Fig. 1: Quadruplex PCR for *C. pseudotuberculosis* species and biovar identification. M: (100bp) DNA Ladder (Stratagene, USA); Lane 1: CpO/S Egy. VSVRI from Sheep; Lane 2: CpO/G Egy. VSVRI from Goat.

Fig. 2: Quadruplex PCR for *C. pseudotuberculosis* species and biovar identification. M: (100 bp) DNA Ladder (Stratagene, USA); Lanes 1: Cp/C Egy. VSVRI from Cattle; Lane 2: Cp/B Egy. VSVRI from Buffalo.

Sequence analysis of *PLD* gene

The Phospholipase D gene (942 bp) was found in the four local *C. pseudotuberculosis* isolates CpO/G_ Egy. VSVRI, CPO /S_ Egy. VSVRI, Cp/C_ Egy. VSVRI and Cp/B_ Egy. VSVRI (Figure 3). The obtained sequence data was submitted into GenBank with accession numbers MN967068, MN867024, MN967069 and MN867025. Alignment of obtained sequences was done with other strains in GenBank from different origins (Table 1). *Corynebacterium pseudotuberculosis* isolates obtained from buffalo and cattle isolates showed high homology percent with biovar equi isolates that range 99.7% −100% on the nucleotide level, while that isolated from sheep and goat were completely identical to each other and with biovar ovis isolates showing 100% homology percent on the nucleotide level. The homology percent between both buffalo and cattle and sheep and goats was 98.7%.

The analysis of the *PLD* gene of *C. pseudotuberculosis* found a fairly conservative nature of biovar ovis isolates (goat and sheep), as the two local isolates clustered with isolates from Brazil, Israel and Australia. Although *C. pseudotuberculosis* biovar equi local isolates from cattle clustered together with isolates from Egyptian buffalo and other equine local isolates from buffalo clustered together with isolates from Belgium, Kenya and USA but all of them come from one branch (Fig. 4). The multiple alignments of Egyptian isolates with *C. pseudotuberculosis* biovar equi and ovis from different origins confirmed that the isolates CP003421.3 and Cp/C Egy. VSVRI has an identical sequence with other equi isolates, but substitution occurs only in amino acid no. 217 (from W to R) and both come from Egypt, one from buffalo and the second from cattle (Fig. 5).

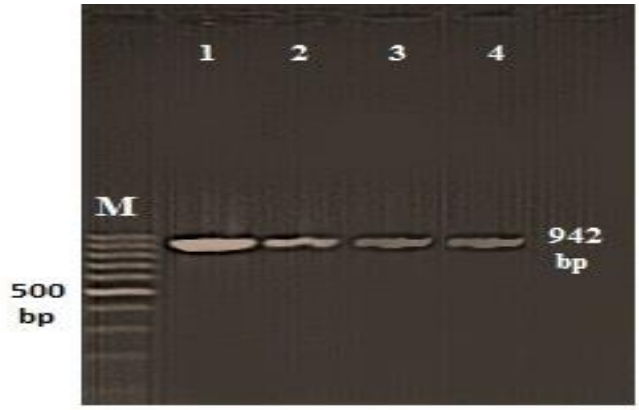

Fig. 3: PCR of *PLD* gene for four local *C. pseudotuberculosis* isolates.
M: (100 bp) DNA Ladder (Stratagene, USA); Lane 1-4. *C. pseudotuberculosis* isolates from Sheep, Goat, Cattle and Buffalo, respectively.

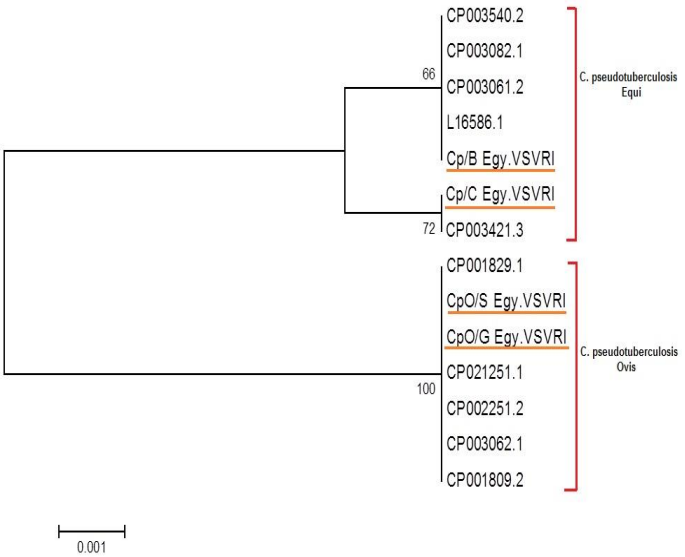

Fig. 4: Phylogenetic tree of the nucleotide sequence of *PLD* gene constructed using Maximum Likelihood method.

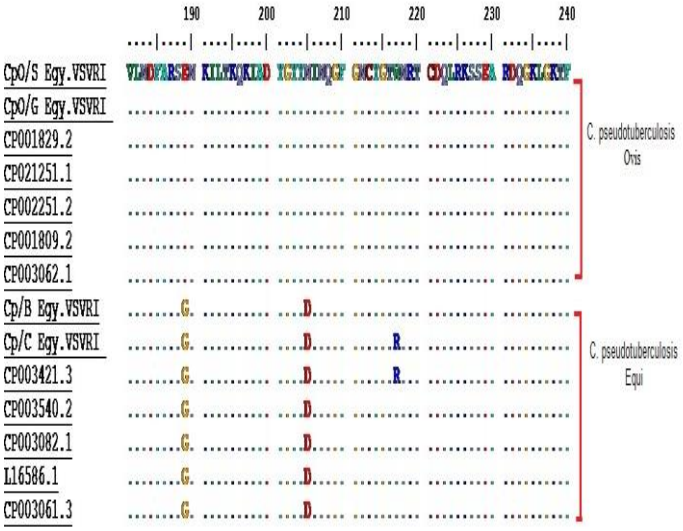

Fig. 5: Multiple alignments of deduced amino acid sequences of *PLD* gene of both ***C. pseudotuberculosis*** ovis and equi.

DISCUSSION

Old diagnosis method based on the isolation and identity of C*orynebacterium pseudotuberculosis* biovars using morphological characters and different chemical techniques such as the nitrate reduction can differentiate between bacterial isolates into nitrate positive (equi) and nitrate negative (ovis). A new method established the insertion of a recent primer set targeting the *narG* gene for multiplex PCR technique **(Pacheco *et al.*, 2007)**; this new tool can identify organisms at biovar and species levels. To our knowledge, the genetic approach is rapid and can clearly differentiate between the two biovars (equi and ovis) of local *C. pseudotuberculosis*.

The multiplex PCR assay much better than the standard biochemical tests, is considered fast and can test large numbers of isolates in a short time **(Songer *et al.*,1988)**. *Corynebacterium pseudotuberculosis* biovars identification relies on the presence or absence and sizes of four PCR DNA products. So, using of Quadruplex PCR assay as a diagnostic tool for isolation and differentiation of *C. pseudotuberculosis* biovars was essential in the laboratory with other standard tools in routine work **(Raoult *et al.*,2004).** Also, Q-PCR can be used directly to diagnose positive clinical samples based on three primers (*16S rRNA, rpoB,* and PLD) **(Pacheco *et al.*,2007)**.

The significance of molecular biology assays helps in distinguishing between the biovars equi and ovis. As shown in the present study, some isolates of *C. pseudotuberculosis* obtained from caprine and ovine failed to reduce nitrate and were negative in the Q-PCR technique. While buffalo and cattle could reduce nitrate by routine biochemical techniques and be positive in Q-PCR. Meanwhile, the biochemical test can distinguish between animal origin biovars **(Oliveira *et al.*,2016 and Roukaya ,1982)**. So, the PCR technique can mainly be used in distinguishing between animal origin biovars as *C. pseudotuberculosis* biovar equi gave +ve narG gene-based PCR.

In contrast, it gave –ve in biovar ovis **(Almeida *et al.*,2015).** All four local isolates were positive for (*16S rRNA, rpoB,* and *PLD*) genes. Until now, the most significant virulence determinant is *the PLD* gene, which is found in all *C. pseudotuberculosis*. So, the differences study of sequence analysis improved the diagnosis and biotyping of local isolates **(Hodgson *et al.*,1990).** After phylogenetic analysis of four *C. pseudotuberculosis*, local isolates were found that the most isolates of biovar ovis clustered together but separately from biovar equi isolates that also clustered amongst them cattle and buffalo, *C. pseudotuberculosis* isolates which showed high homology percent with biovar equi isolates that range 99.7% −100% on the nucleotide level, while that isolated from goat and sheep were completely identical to each other and with biovar ovis isolates showing 100% homology percent on the level of nucleotide.

The homology percent between both buffalo, cattle and goat, sheep was 98.7%. It was observed that using phylogenetic analysis ,at the genetic level, the two biovars are different. The differences in the clustering pattern of *C. pseudotuberculosis* biovar equi and biovar ovis isolates could reflect the number of specific genes in each biovar **(Soares *et al.*,2013)**, which occur due to genetic variation between them **(Almeida *et al.*,2015)**.

CONCLUSION

It can be concluded that, the Quadruplex PCR is reliable in the identity of *C. pseudotuberculosis* biovars in microbiological laboratories. Sequence and phylogenetic analysis of the PLD gene of the four isolates of the two biovars (ovis and equi) have confirmed the results obtained by Q-PCR suggesting that the Q-PCR with the four genes used in our study was rapid and accurate tool for *C. pseudotuberculosis* characterization.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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