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Characterization of Virulence Genes Present in *Corynebacterium pseudotuberculosis* Strains Isolated From Buffaloes

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Abstract: Forty aspirates collected from swellings of buffaloes showing clinical symptoms of the Oedematous Skin Disease were examined bacteriologically, biochemically and by uniplex and multiplex PCR for virulence genes screening. Ten isolates were identified as *Corynebacterium pseudotuberculosis* in a percentage of (25%) and all isolates exhibited a synergistic hemolytic activity with *Rhodococcus equi* culture filtrate and inhibited staphylococcal hemolytic activity. The presence of universal 16s gene of *C. pseudotuberculosis* was confirmed by uniplex PCR in all 10 isolates. Application of multiplex PCR was done and all isolates of *C. pseudotuberculosis* were positive for presence of *pld* gene of *Corynebacterium pseudotuberculosis* while only 5 isolates were positive for presence of *pld* gene of *Corynebacterium ulcerans* and 7 isolates were positive for presence of diphtheria toxin *dt* gene of *Corynebacterium ulcerans*.

Key words: *Corynebacterium pseudotuberculosis* · Phospholipase D (PLD) · Virulence Genes · Oedematous Skin Disease (OSD)

INTRODUCTION

Corynebacterium pseudotuberculosis is a Gram-positive, non-spore forming; facultative intracellular bacterium classified into 2 biotypes based on host preferences and nitrate-reducing activity [1, 2]. *C. pseudotuberculosis* is the causative agent of "Oedematous Skin Disease (OSD)" an endemic disease of buffaloes in Egypt. It appears as outbreaks during the summer months especially in Lower Egypt which is characterized by high humidity and usually associated with the breeding season of the blood sucking fly *Hippobosca equine*; the main transmitter of the causative agent [3]. Although mortality is low, morbidity is high and the treatment of diseased animals may extend for months causing economic losses to farmers due to reduction of animal productivity; decrease in work efficiency of the animal, expensive medicaments and surgical intervention in some cases which represent a noxious situation for owners and veterinarians [4].

It is well documented that *C. pseudotuberculosis* biotype 2 (nitrate positive), exerts its pathogenesis by secretion of exotoxin (s) including phospholipase D (PLD) which has been implicated as the major virulence factor and incriminated as main player of pathogenesis of OSD [1]. In spite of both biotype 1 (nitrate negative) the causative agent of caseous lymphadenitis (CLA) in sheep and goat and biotype 2 (nitrate positive) produce PLD, there is a difference in pathogenicity for guinea pigs, whereas, biotype 2 shows more rapid and reverse hemorrhagic lesions at site of inoculation associated with rapid death of the experimental animals and this may be attributed to the presence of toxigenic factor(s) beside PLD [2]. There is a lack of information about the virulence factors of *C. pseudotuberculosis* and the pathogenic mechanisms of the diseases other than CLA caused by this bacterium [5]. The observations of severe toxic activity of buffalo strains may be attributed to the difference of antigenic structures of both biotypes and may be consequently that there are other virulence factors in addition to PLD toxin and one of the predicted toxins is the diphtheria toxin (DT) (tox) which may be produced by sporadic isolates of biotype1 and closely related type of Corynebacterium known as *Corynebacterium ulcerans* [6].

Despite the various molecular strategies that have been employed, efficient tools for the genetic study of *C. pseudotuberculosis* isolated from buffaloes in Egypt

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are still scarce. In fact, the main reason for the lack of molecular investigation of this organism is that the genetics of the genus have been little studied with modern techniques, making it difficult to identify and characterize factors that could be involved in virulence.

The present study was conducted to give a chance for investigation of the virulence factors' genes in *C. pseudotuberculosis* strains isolated from buffalo in Egyptian farms.

MATERIALS AND METHODS

Forty aspirates were collected from closed oedematous skin lesions of water buffaloes showing clinical symptoms of the Oedematous Skin Disease. After disinfecting the surface using 5% tincture iodine, the aspirate were separately collected properly labeled and taken in a cold container to the laboratory and streaked onto brain heart infusion agar plates containing fosfomycin and nalidixic acid and incubated at 37°C for 24-48 hours. Films were prepared from suspected *Corynebacterium pseudotuberculosis* colonies and stained with Gram's stain.

Biochemical Identification: Colonies identified as *C. pseudotuberculosis* according to cultural and morphological examination were subjected to the biochemical tests as prescribed by Koneman *et al.* [7] and Collee *et al.* [8].

Hemolytic Activity: Preparation of *Rhodococcus equi* culture filtrate [9].

Preparation of *C. pseudotuberculosis* culture filtrate [10].

Culture of *C. pseudotuberculosis* was grown in brain heart infusion broth to which 0.1% Tween 80 was added and incubated for 72 hours with continuous shaking. Then bacterial cells were pelleted down at 15000 Xg and the supernatant was taken and filtered through membranes of 0.45µ Millipore filter.

Synergistic hemolysis by PLD of *C. pseudotuberculosis* and *Rhodococcus equi* factor:

The obtained *C. pseudotuberculosis* isolates were screened for their PLD activity by detection of their synergistic hemolysis with *R. equi* filtrate using sheep erythrocytes by hemolytic assay of *C. pseudotuberculosis*, "Modified CAMP" [11].

Antagonistic hemolysis of *C. pseudotuberculosis* with *Staphylococcus aureus* " Reverse CAMP" [12].

Extraction of DNA Template from *C. Pseudotuberculosis* Isolates: Genomic DNAs of *C. pseudotuberculosis* isolates were extracted by using an extraction kit (QIA amp mini kit, Qiagen), according to the kit instructions.

Polymerase Chain Reaction for Identification of *C. pseudotuberculosis* Universal 16s Gene: Primers targeting the 16S rRNA gene of *C. pseudotuberculosis* were obtained according to Cetinkaya *et al*. [13] as demonstrated in Table (1).

Multiplex polymerase chain reaction (multiplex PCR) was carried out according to Luis *et al*. [14].

The oligonucleotide primers used in this reaction are listed in Table (2), all reactions were carried out in a final volume of 50 µl in PCR tubes. The reaction mixture consists of 5 µl of the extracted DNA template from the bacterial cultures, 5 µl of 10x PCR buffer, (75 M Tris HCl

Table 1: Primers targeting the 16S rRNA gene of C. pseudotuberculosis

Primers of *C.pseudotuberclusis* 16s rRNA	Primers sequence (5' to 3')	PCR product
16S-F	ACCGCACTTTAGTGTGTGTG	816 bp
16S-R	TCTCTACGCCGATCTTGTAT	

Table 2: Oligonucleotide primers used for plds and dt

Primers of *C.pseudotuberclusis pld* p	Primers sequence (5' to 3')	PCR product
Sense primer PLDp	ATTATGGGGATGCTTC	930bp
Anti sense primers PLDp	TCACCACGGTTATCCGCT	
Primers of *C. ulcerans pld* u		
Sense primer PLDu	GTGGAAGACAATCACTTCTCGACC	404bp
Anti sense primers PLDu	GCTTATCTACCCACGTCTTGATG	
Primers of *C. ulcerans dt*		
DT1 (upper strand)	GTTTGCGTCAATCTTAATAGGG	248bp
DT2 (lower strand)	ACCTTGGTGTGATCTACTGTTT	

PH9.0, 2mM MgCl2, 50 mM Kcl, 20 mM(NH4) 2So4),1 µl dNTPS (40µM), 1µl(1U Ampli Taq DNA Polymerase) and 1µl from the forward and reverse primers of PLDp, PLDu, DT1primer and DT2primer. All primers were used together and volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil wax was added and the thermal cycler was adjusted as following program: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing step at 55°C for 1 minute and extension at 72°C for 1 minute. A final extension step was done at 72°C for 7minutes.

The PCR products were electrophoresed in 1.5% agarose gel using Tris-acetate EDTA buffer. The gel containing separated DNA was stained with ethidium bromide. Standard marker containing known fragments of DNA of 100 bp ladder was used (Fermentas Life Science, EU).

RESULTS

Out of 40 aspirates collected from closed oedematous skin lesions of water buffaloes, a number of 10 bacterial isolates were identified as *C. pseudotuberculosis* in a percentage of (25%) based on cultural, morphological and biochemical characters. All isolates are Gram-positive, non-sporulated, non motile pleomorphic, curved rods, catalase, urease and nitrate reduction positive which is specific reaction which differentiate the biotype 2 (buffalo origin) from biotype 1(sheep origin). The bacteria ferment glucose and fructose but not ferment galactose, sucrose, lactose, mannitol, or xylose.

Synergistic Hemolytic Activity of *C. pseudotuberculosis* and *Rhodococcus equi* Culture Filtrate (Modified CAMP): All the *C. pseudotuberculosis* isolated from buffaloes tested in this study showed synergistic hemolysis with *Rhodococcus equi* culture filtrate when streaked onto LB agar containing 5% sheep erythrocytes and 10% *Rhodococcus equi* culture filtrate. Clear zone of hemolysis surrounded the tested colonies as their diameter and intensity of which depend upon the extent of toxin production of the unknown isolate as shown in Figure (1).

Inhibition of Staphylococcal Hemolytic Activity with PLD (Reverse CAMP Test): The exotoxin producer strain of *C. pseudotuberculosis* inhibited the hemolytic activity of vertical streaked colonies β hemolysin produced by staphylococci that appear as a wide clear zone of growth

Fig. 1: Synergistic hemolysis activity of *C. pseudotuberculosis* colonies and R. equi culture filtrate

Fig. 2: Inhibition hemolysis activity of *Staphylococcus aureus* by *C. pseudotuberculosis* colonies.

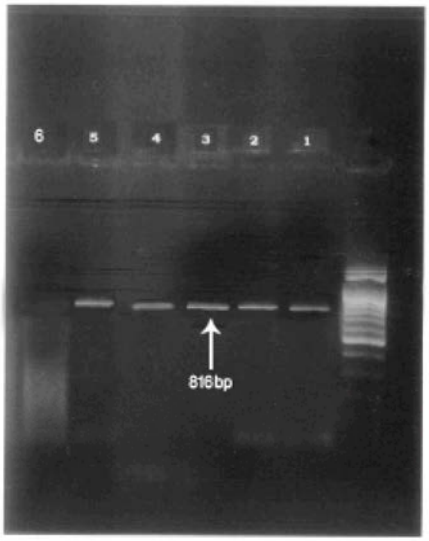

Fig. 3: Lanes from 1,2,3,4 and 5 showed positive bands of 816 bp specific to universal 16s gene of *C. pseudotuberculosis*.

Lane 6 represented negative control.

The DNA molecular weight marker (100bp ladder)

Multiplex polymerase chain reaction (multiplex PCR):

inhibitor between the two streaked strains as shown in Figure (2). All corynebacterial buffalo isolates were inhibitors for β staphylococcal hemolytic activity in a percentage of 100%.

Detection of Universal 16s Gene of *C. pseudotuberculosis* in Buffalo Isolates with Polymerase Chain Reaction: Products of the expected size (816 bp) were successfully obtained with DNA templates of *C. pseudotuberculosis* isolates of buffalo origin.

All 10 isolates showed positive results for presence of universal 16s gene detected with PCR (Figure 3).

DISCUSSION

Corynebacterium pseudotuberculosis is a Gram-positive and facultative intracellular bacterium that does not sporulate, has fimbriae and displays pleomorphic cells which vary from coccoid to filamentary rods. Two biovars have been described for *C. pseudotuberculosis*, ovis (biotype1) and equi (biotype2); they are mainly distinguished by their ability to reduce nitrate and tropism for infecting particular kinds of animals [1]. It is known that *C. pseudotuberculosis* (biotype2) causes "Oedematous Skin Disease (OSD)" among buffaloes, an acute disease manifested by superficial swelling which culminate in the formation of abscesses and release of serous bloody exudates. This picture of the disease is different from that occurs in sheep (chronic lymphadenitis) caused by the same organism but biotype1 [15].

The results obtained from isolation and identification showed that out of 40 aspirates collected from closed oedematous skin lesions of water buffaloes, a number of 10 bacterial isolates were identified as *C. pseudotuberculosis* in a percentage of (25%), this result exactly agrees with that obtained by Selim [16] who isolated 2 strains of biotype 2 from exudates of subcutaneous abscess of buffaloes aged 1-6 years by the ratio of 25% and near the results of Mohammad [17] and Syame [18] who isolated *C. pseudotuberculosis* from skin lesion of buffaloes by ratio 14. 3 and 14% respectively. High results differ was stated by Zaki, [19] who isolated *C. pseudotuberculosis* from samples collected from oedematous lesions in buffalo at El-Minia Governorate by a ratio of 41.5%.

The data of biochemical identification revealed that all isolates were positive for catalase, urease and nitrate reduction, ferment glucose and fructose but not ferment galactose, sucrose, lactose, mannitol, or xylose and these data agree with that reported by Fernanda *et al*. [20].

Concerning their biological activities, all isolates were subjected to modified CAMP and reverse CAMP tests and all isolates showed large hemolytic zones around the growing colonies in the presence of *Rhodococcus equi* and inhibit the hemolytic activity of *S. aureus* β lysine respectively as shown in Figures (1) and (2) and these results agree with Songer *et al*. [11] and Egen *et al*. [12].

Molecular methods, including nucleic acid hybridization and 16S rRNA gene sequence analysis, have been used to determine the degree of relatedness of many different corynebacterial species and strains [21]. Figure (3) showed the identification of isolates was confirmed by detection of 16s rRNA gene by PCR and all isolates were positive and showed amplification of specific bands at 816 bp molecular weight and agree with the data stated by Khamis *et al.* [22] and Takahashi *et al*. [23].

Virulence factors play an important role in the adhesion, invasion, colonization, spread inside the host and immune system evasion of pathogenic bacteria; they also allow contact, penetration and survival inside the host [24]. It is somewhat known that virulence factors of *C. pseudotuberculosis* are not declared up till now, although phospholipase –D is well established as the major virulence factors in *C. pseudotuberculosis* [25].

Phospholipase D (PLD) has for some years been implicated as the major virulence factor of *C. pseudotuberculosis* [26]. It promotes the hydrolysis and degradation of sphingomyelin in endothelial cell membranes, which increases vascular permeability and contributes to the invasion, spread, dissemination and persistence of the bacterium in the host [24, 26, 27]. Beside that enabling the bacteria to escape from neutrophils and impairing neutrophil chemotaxis toward the site of infection so it protects the organism from killing by phagocytic cells [28] and encoded by pld gene [29].

All isolates were investigated for phospholipase –D activity by modified CAMP and reverse CAMP and showed positive result, this leads to that PLD is a characteristic product of *C. pseudotuberculosis* isolates.

The multiplex PCR was done for all isolates of *C. pseudotuberculosis* and Figure (4) demonstrated that all isolates were positive for amplification of the specific nucleotide base (930 bp) size of *pld* gene of *C. pseudotuberculosis* and this agree with Barksdale *et al*. [30] who stated that PLD probably is characteristic of most or almost all strains of *C. pseudotuberculosis*, only 5 isolates were positive for amplification of the specific nucleotide base (404bp) size of pld gene of *C. ulcerans* while 7 isolates were positive for amplification of the specific nucleotide base (248 bp) size of diphtheria toxin dt gene of *C. ulcerans*.

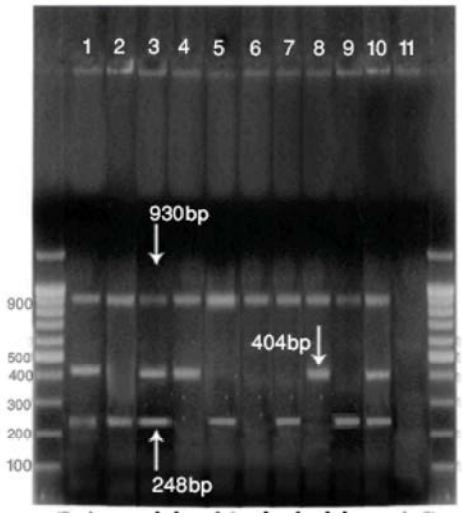

Fig. 4: Shows that all buffalo isolates of *C. pseudotuberculosis* are positive for presence of pld gene of *C. pseudotuberculosis* detected with PCR of products of the expected size (930bp), only 5 isolates are positive for presence of pld gene of *C. ulcerans* with the size of (404bp) and 7 isolates are positive for presence of diphtheria toxin dt gene of *C. ulcerans* with the size of (248 bp)

Lanes from 1-10 showed positive bands of 930bp specific to pld of *C. pseudotuberculosis*.
Lanes 1,3,4,8 and 10 showed positive bands of 404bp specific to pld of *C. ulcerans*.
Lanes 1,2,3,5,7,9 and 10 showed positive bands of 248bp specific to diphtheria toxin of *C. ulcerans*.
Lane 11 represented negative control.

This result coincide with Wong and Groman [31] who could detect DT produced from 2 strains isolated from Egyptian buffaloes and Christopher *et al*. [32] who reported many of *C. pseudotuberculosis* isolates produce a toxin which give a line of identity with diphtheria toxin (DT) in gel immunodiffusion tests. Diphtheria toxin is known to contribute to disease progression, causes cell death by inhibiting protein biosynthesis, [33, 34].

Also the results revealed that there are 3 isolates have the 3 virulent genes and 6 isolates have 2 of the virulent genes while one isolate had the pld gene of *C. pseudotuberculosis* only.

The previous results suggested that the *C. pseudotuberculosis* isolates obtained from buffaloes tend to carry more than one virulent gene so can produce several types of toxins having synergistic or cumulative action leading to the acute form of the disease and their rapid lethal effect on the laboratory animals and the severity of virulence depends upon the number of factors in the same strain which may explain the variety in clinical picture of the OPD.

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