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SYNERGISTIC HAEMOLYTIC ACTIVITY AND ITS CORRELATION TO PHOSPHOLIPASE D PRODUCTIVITY BY *CORYNEBACTERUIM PSEUDOTUBERCULOSIS* EGYPTIAN ISOLATES FROM SHEEP AND BUFFALOES

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ABSTRACT

Fourteen isolates of *Corynebacteruim pseudotuberculosis* of them 7 were isolated from sheep with Caseous Lymphadenitis "biotype 1" and 7 isolated from buffaloes with Oedematous Skin Disease "biotype 2". All isolates were identified by standard microbiological techniques and by polymerase chain reaction targeting, 16S rRNA and *phospholipase D* genes. Synergistic haemolytic titers of all isolates were assayed by plate technique. The presences of *phospholipase D* gene in supernatants of all isolates were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis immunoblot technique by using hyperimmune serum raised in rabbit immunized with recombinant *phospholipase D* gene antigen. The concentration of *phospholipase D* gene was assayed by scanning the bound *phospholipase D* gene with specific antibodies that appeared at 31.5 kDa. Results presented that there is no correlation between titer of Synergistic haemolytic activity and the actual *phospholipase D* genes concentration in culture supernatants. Also results presented that Synergistic haemolytic activity and *phospholipase D* genes produced by biotype 2 (buffalo isolates) was generally higher than those by biotype 1(sheep isolates).

Key words: *Corynebacteruim pseudotuberculosis*, Oedematous Skin Disease, Caseous Lymphadenitis, recombinant PLD, Synergistic haemolysis.

INTRODUCTION

Phospholipase D (PLD) is a potent exotoxin produced by *Corynebacteruim pseudotuberculosis* of sheep origin (9, 11, 26) and by buffalo isolates (8). PLD is a secreted exotoxin that possesses sphingomyelinase activity and has been shown to increase vascular permeability IN vivo (2, 30), exhibit synergistic haemolysis (SH) of sheep blood cells in the presence of products from *Rhodococcus equi* and reduce the viability of ovine neutrophilis (2). Depending upon the information that PLD is the major virulence factor in *C. pseudotuberculosis* , many significant efforts have been made to produce effective caseous lymphadenitis (CLA) vaccines. The majority of prepared vaccines were derived from PLD-rich culture supernatants, inactivated with formalin to produce toxoid vaccine (6, 20, 21, 29). But field application of these

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toxoid vaccines showed controversy character while Eggleton *et al.* (7) were satisfactory about protective efficacy of PLD toxoid vaccines. Other researchers reported that toxoid vaccines and inactivated corynebacterial cells (bacterins) provide partial protection (3).

The reason of inadequate toxoid vaccines is still obscure, although Paton *et al.* (18) attributed this low effective protection to the inappropriate use of the vaccine and reported that although 43% of the farmers applied commercial CLA vaccines, only 12% used them correctly. One of the propositions that may help in explanation of the inadequacy of some toxiod vaccines may be attributed to variation in concentration of PLD included in culture supernatants used for preparation of the toxoid vaccine.

Concentration of PLD in culture supernatants is evaluated by indirect methods depending upon its synergistic haemolytic activity. They considered the increase in SH activity is correlated to the concentration of PLD included in culture supernatants, although there is still no definitive evidence that PLD and SH activity are one and the same (6). The fixed optimal amount of antigen in any vaccine is an important factor in preparation of vaccine, this condition cannot be guaranteed in toxoid vaccines in which the PLD antigen is measured by SH activity of culture supernatants.

The correlation between SH activity and the actual concentration of PLD included in culture supernatants has not been previously explored. The present investigation was undertaken to evaluate the correlation between SH activity and the actual concentration of PLD involved in culture supernatants of *C. pseudotuberculosis* obtained from sheep with Caseous Lymphadenitis (CLA) and buffaloes with Oedematous Skin Disease (OSD).

MATERIALS AND METHODS

Clinical specimens

Fourteen pus samples were collected aseptically from abscessed lymph nodes of naturally infected sheep (*n*=7) and buffaloes (*n*=7) found in two CLA- OSD endemic areas of Egypt. Microbiological examinations, followed by biochemical identification, were used as a gold standard to confirm infection with *C. pseudotuberculosis*. In brief, bacteriological cultures were made of pus specimens and the resultant *C. pseudotuberculosis*-resembling colonies that stained Gram-positive were tested further for biochemical properties (glucose fermentation, urease and catalase) (7, 31). Synergistic haemolysis with *Rhodococcus equi* ATCC 33701 and inhibition of *ß*-haemolysis by *Staphylococcus aureus* ATCC 25923 were also evaluated (7, 31).

Bacterial strains and culture condition

The study was undertaken with 7 isolates recovered from buffaloes infected with OSD and 7 isolates recovered from sheep infected with (CLA). Isolates were cultured initially into brain heart agar supplied with fosfomzein and nalidixic acid (31).

Biotypes were determined by conventional tests as described previously (1) in addition to starch hydrolysis.

Starch agar hydrolysis test

It is prepared as in welcome to microbugs starch agar medium with some modification. Briefly the differential medium was prepared by suspension of 25 g of starch powder in 1 L of purified water, mixed thoroughly and boiled for 1 min to completely dissolve the powder, then 15 g of brain heart agar (Oxoid ®) were added, mixed thoroughly, then autoclaved at 121°C for 15 min. Isolates were streaked on plates of starch agar and incubated for 48 hours at 37°C. The surfaces of inoculated media were flooded with Gram iodine. Starch hydrolysis was indicated by the development of a clear zone around the colonies against a dark blue back ground.

DNA isolation: Two different protocols were adapted for extracting DNA from pure bacterial cultures and clinical samples.

Bacterial cultures: Chromosomal DNA extraction from bacterial strains was carried out according to the standard protocol of Sambrook *et al.* 22 with some modification .The DNA concentrations was determined spectrophotometrically.

Clinical samples: 100 mg pus was resuspended in 1 ml TE/lysozyme. Samples were incubated for 1 h at 37 °C; 20 µl proteinase K (20 mg ml^{-1}; Invitrogen) was added, followed by incubation for 2 h at 56 °C. Samples were divided into two aliquots of 500 µl, and 25 µl 30 % (w/v) sarcosyl was added to each; mixtures were incubated for 20 min at 65 °C and then for 5 min at 4 °C. DNA was purified and precipitated as described above.

Primers and PCR conditions

The oligonucleotide primers used in this study were designed to detect 16S rRNA and PLD genes of *C. pseudotuberculosis*. Both of them were obtained from previously published work (4,13,17). The oligonucleotide primers specific for 16S rRNA; 16S-F 5' ACC GCA CTT TAG TGT GTG TG3' and 16S-R 5' TCT CTA CGC CGA TCT TGT AT 3' could amplify 816 base pair fragments. The oligonucleotide primers specific for PLD genes of *C. pseudotuberculosis*; *PLD* F5': CGG CCC GGG ATT ATG GCG ATC ATG CTT C3' and *PLD* R5': CGC AAG CTT TCA CCA CGG GTT ATC CGC T 3' could amplify 930 base pair fragments. The PCR reactions will carry out according to Cetinkaya et al.,4.

Anti-recombinant phospholipase D hyperimmune sera

A highly specific rabbit hyperimmune serum was prepared by inoculation of rabbits with recombinant PLD (rPLD) antigen prepared and provided from Biotechnology Center of Veterinary Services and Researches (BCVSR) Cairo University (8). Three Boscat rabbits weighing 2.5 Kg were inoculated subcutaneously with 1 ml dose containing 25 µg rPLD protein mixed with complete Freund's adjuvant (Sigma) followed by 2 doses of 25 µg rPLD mixed with incomplete Freund's adjuvant (Sigma) at weekly intervals. One week post last dose antibodies were assayed by ELISA (23) using rPLD protein as a coating antigen. Titers were expressed as the reciprocal of the dilution which gave an OD three fold above the OD of preimmune serum analysed on the same plate. Titers obtained from immunized rabbits were more than 128 and sera were pooled, aliquoted and stored in – 20°C.

Selective media for obtaining maximum yield of PLD

C. pseudotuberculosis loses its power of PLD production under laboratory storage conditions and it needs reactivation process during subculturing from the stock cultures. To guarantee the maximum yield of PLD produced by stock cultures of *Corynebacteria*, we used the 2 stages media recommended by Soheir (24) that activate stock culture to produce maximum amounts of PLD.

The first stage of cultivation was performed by inoculation of bacteria into cooked meat medium (Oxoid ®) and incubated at 37°C for 24 hours, then the whole constituents of incubated culture were transferred into flasks that contain brain heart broth (Oxoid ®) supplied with 0.1% v/v Tween 80 and 3% glucose in a percent of 1 part cooked meat medium to 3 parts of BH broth. Mixed media were incubated in shaker incubator for 48 hours at 37°C.

Titration of synergistic haemolytic activity of culture filtrates

Synergistic haemolytic (SH) activity of culture filtrates (CF) that rich in PLD was titrated by plate method according to Tachedjian *et al.* (27).

Briefly in 96 round button plates 200 µl of filtered culture supernatant were dispensed in the first well and 50 µl of sterile PBS to subsequent wells, taken 150 µl from the first well were mixed with the second well and so on in subsequent wells. To each well defibrinated sheep blood and filtered *Rhodococcus equi* culture supernatants were added to all wells in a final concentration of 1.5% and 13.5%, respectively. Plates were

incubated overnight at 37°C and titers were red as the last well to exhibit complete haemolysis.

Electrophoresis and immunoblotting

Total proteins in each culture filtrate were measured by Lowry *et al*. (12), then concentrated to 1/20 of the original volume (1 ml to 50 µl) by using the dry vacuum concentration (Speedvac System-Savant # SS11).

Each concentration sample was treated with reducing buffer (Tris 91 g, SDS 1%, distilled water 500 ml) in the ratio of 1:1; the treated samples were immersed in a boiling water bath for 2 minutes to ensure protein denaturation. Electrophoresis was performed (10). Briefly 10 µl of each treated concentrate were loaded into each lane and electrophoresis was done for 4 to 6 hours at 100 volt. Gels were fixed overnight in 50% ethanol and 5% glacial acetic acid, followed by staining with 0.25% commasie dissolved in destaining solution (45% methanol, 5% glacial acetic acid, 50% distilled water) for 1 hour, followed by destaining of the gels till the background become completely clear. Proteins in some unstained gels were electrotransferred to nitrocellulose membranes (28). Membranes that were blocked with blocking buffer (5% bovine serum albumin in 0.3% PBS-Tween, pH 7.2) for 2 hours were washed in washing buffer and spliced into strips. In this investigation, we used highly purified recombinant PLD produced in BCVRS by Ghoneim *et al.* (8). The rPLD antigen was used for preparation of highly specific hyperimmune serum against PLD. This rabbit hyperimmune serum was used for binding to specific electrophorotic bands resulted in SDS-PAGE immunoblot technique

The strips were exposed to diluted rabbit hyperimmune serum (1:50) and incubated for 1 hour at 25°C. The strips were washed 3 times (5 minutes each) in washing buffer and then were exposed to goat antirabbit IgG peroxidase conjugate (Sigma) diluted 1:1000. Bound antibody was visualized by use of 4-chloro-1-naphthol/H_2O_2 (0.5 mg /ml/0.15% in PBS with 17% methanol) as substrate. Inoculation with substrate was in dark at 25°C for 45 minutes.

Analysis of immunoblotted bands

PLD protein in bound bands was assayed by Gel-Pro-program (USA).

RESULTS

Characteristics of strains

The pattern of diagnostic tests for 14 strains revealed 7 strains of *C. pseudotuberculosis* of sheep origin and 7 strains of buffalo origin. Results in Table 1 revealed that the two biotypes could only be distinguished by nitrate reduction test and starch hydrolysis. *C. pseudotuberculosis* biotype 2 is nitrate positive and could hydrolyse starch. Moreover, it had been noticed that the two major criteria which are nitrate reduction and starch hydrolysis can distinguish these organisms as shown in Figure 1.

Table 1: Diagnostic reaction of *C. pseudotuberculosis* of sheep origin and buffalo origin

Test	Sheep isolates	Buffalo isolates
Sugar fermentation		
- Glucose	7/7	7/7
- Maltose	7/7	7/7
- Fructose	7/7	7/7
- Sucrose	0/7	0/7
Starch hydrolysis	0/7	7/7
Trehalose	0/7	0/7
Urease production	7/7	7/7
Nitrate	0/7	7/7
SH	7/7	7/7
16 rRNA	7/7	7/7
pld gene	7/7	7/7

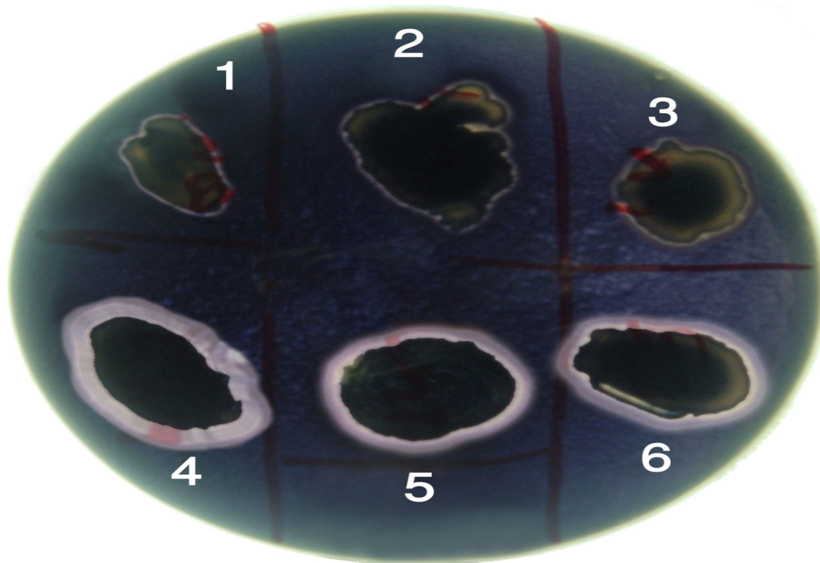

Figure 1: Starch hydrolysis plate showing clear zone of hydrolysis around colonies of *C. pseudotuberculosis* of buffalo origin Number. (4, 5, 6) and sheep isolates Number (1, 2, 3) showing no hydrolysis.

Characterization of the isolates using PCR

Amplification of 816 bp fragments specific for 16S rRNA of *C. pseudotuberculosis* were observed with all strains collected from sheep and buffaloes as shown in Figure 2. Moreover, amplification of 910 bp fragments specific for *PLD* gene were also observed with all sheep and buffaloes strains of *C. pseudotuberculosis* as shown in Figure 3.

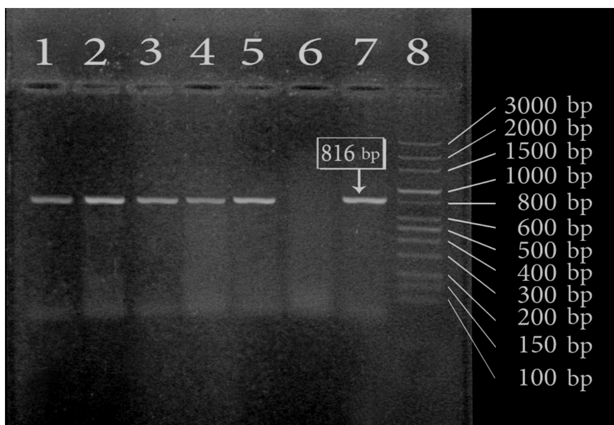

Figure 2: Agarose gel electrophoresis showing amplification of 816 bp fragments specific for 16S rRNA of *C. pseudotuberculosis*. While lane 6 showing negative control.

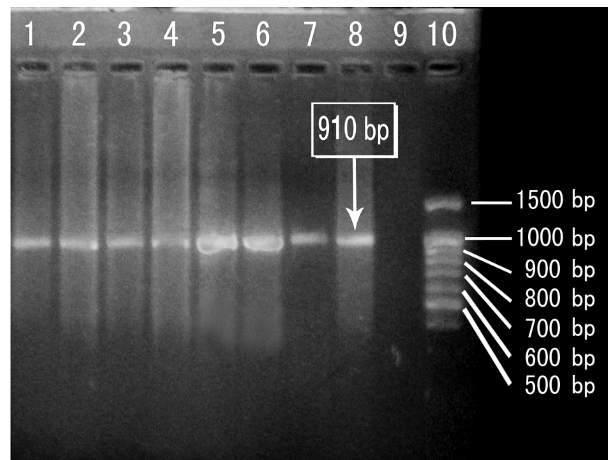

Figure 3: Agarose gel electrophoresis showing amplification of 910 bp bands specific for PLD gene from 4 sheep isolates no. (1,2,3,4) and 4 buffalo isolates No. (5,6,7,8) of *C. pseudotuberculosis*. Lane 9 negative control.

Maximum yield of PLD produced by isolates of *C. pseudotuberculosis*

All isolates revealed SH activity by streaking on BH agar media and all isolates revealed synergistic haemolytic zones around streaked colonies with varying zone of haemolytic

reaction, but all buffalo isolates showed the wider zones of haemolysis if compared with sheep isolates. The titers of haemolytic activity of *C. pseudotuberculosis* and the SH activity of both sheep and buffalo isolates are shown in Table 2.

Table 2: Synergistic haemolytic activity of *C. pseudotuberculosis* of sheep and buffalo isolates measured by haemolysis zone diameter

Origin of isolates	No. of isolate	Haemolytic zone	Haemolytic titer	Concentration of PLD in µg/ml
Sheep	1	+ + + +	$(1.33)^7$	18.30
	2	+ + + +	$(1.33)^7$	9.32
	3	+ +	$(1.33)^4$	13.40
	4	+ + +	$(1.33)^3$	19.40
	5	+ +	$(1.33)^6$	15.20
	6	+ +	$(1.33)^5$	15.90
	7	+ +	$(1.33)^3$	16.20
Buffalo	1	+ + + +	$(1.33)^6$	21.20
	2	+ + + + +	$(1.33)^5$	19.50
	3	+ + + + +	$(1.33)^7$	23.90
	4	+ + + +	$(1.33)^7$	23.10
	5	+ + + +	$(1.33)^7$	18.20
	6	+ + +	$(1.33)^4$	19.1
	7	+ + + +	$(1.33)^6$	21.20

Haemolytic zone diameter: + + + + + very strong haemolysis
+ + + + strong haemolysis
+ + + moderate haemolysis
+ + weak haemolysis
+ very weak haemolysis

Detection of PLD concentration in culture filtrates of *C. pseudotuberculosis* of sheep and buffalo origin by SDS-PAGE and immunoblot technique

Results in Table 2 show that buffalo isolates produced PLD in concentration range from 18.2 µg /ml up to 23.9 µg /ml, while sheep isolates produced PLD in amounts ranged from 9.32 µg/ml up to 19.4 µg/ml. It can be observed that the highest concentration of PLD (19.4 µg/ml) was produced by sheep strain showing the least titer of SH activity $(1.33)^3$. Also in buffalo isolates, it can noticed that strain No. 6 with haemolytic titre of $(1.33)^4$ produced PLD in concentration of 19.1 µg/ml in comparison to strain No. 5 which produced higher titer of SH activity $(1.33)^7$ but lower PLD productivity (18.2 µg/ml). Also it can be observed that all isolates that revealed the same titer of SH activity $(1.33)^7$ produced variable amounts of PLD 23.9 µg/ml, 23.1 µg/ml and 18.1 µg/ml from buffalo isolates No 3, 4, 5, respectively as shown in Table 2.

DISCUSSION

To assess the SH of the two biotypes of *C. pseudotuberculosis* and its correlation to the actual concentration of PLD in the culture supernatants, 7 isolates (biotype 1) were collected from sheep showing clinical symptom of CLA and 7 isolates (biotype 2) were collected from buffaloes diseased with OSD. During our investigation about characterization of *C. pseudotuberculosis* of sheep origin (biotype 1) and of buffalo origin (biotype 2), we noticed that, during investigation of SH activity of both biotypes, the stock culture of *Corynebacteria* revealed lower SH activity if compared with recently isolated strains and the extent of decrease that is reversibly related to the period of storage of the isolates which may be attributed to the decrease of PLD production by stock cultures. Moreover, many commercial toxoid vaccines depend upon measuring the SH activity as indicator for the concentration of PLD secreted into culture supernatants, which needs accurate evaluation of the correlation between SH activity and the indeed concentration of PLD included in supernatants used for preparation of toxoid vaccines. Purification of PLD to homogeneity is a necessary prerequisite to molecular investigation of the enzyme and each extractivity to it including it's SH activity. Purification to near homogeneity has been reported, but it was a method that yields small amounts of enzymes. We need a purification method that

would allow us to process large volumes of culture supernatant fluid and yield nearly homogenous PLD (6).

All buffalo isolates showed higher SH activity if compared with sheep isolates (biotype 1) as shown in Table 2. It can be collectively observed that buffalo isolates are more potent in SH activity if compared with biotype 2 (sheep isolates). The reason is not clear but it can be proposed that biotype 2 (buffalo isolates) may produce other hemolytic factors beside PLD that increase the extent of SH activity.

The resulting correlation between the extent of SH activity and actual contents of PLD in supernatants of biotype 1 and biotype 2 isolates is shown in Table 2. It can be noticed that sheep isolate (No.1) had strong SH activity titer and produced high yield of PLD in culture f supernatants, while isolate (No. 2) having the same high titer of SH but produced half the amount produced by isolate No. (1). at the same time, strain No. (6) showing less SH titer and produced high concentration of PLD in culture supernatants. The same poor correlation between SH activity and actual amounts of PLD in culture supernatants is observed in strains of biotype 2 (buffalo isolates). Isolate (No. 1) having the least titer of SH activity and produced PLD in large amounts. Moreover, biotype 2 isolates No. 3, 4, 5 had the highest SH titer, but they produced variable amounts of PLD enzyme in culture supernatants 23.9, 23.1, 18.2 µg/ml, respectively. The obtained results indicate a poor correlation between SH activity of C. *pseudotuberculosis* isolate and production of PLD enzyme in culture supernatants. Poor correlation was also reported by previous investigation, Muckle and Gyles, (14, 15) reported a poor correlation between PLD enzyme contents in supernatants of *C. pseudotuberculosis* measured by radiometric assay. Egen *et al.* (6) reported that isolates having 3 fold variations in PLD concentration measured by radiometric assay produced nearly identical areas of haemolysis on *Rhodococus* blood agar plates.

The explanation of the reason of poor correlation between SH activity and the actual contents of PLD in supernatants of *C. pseudotuberculosis* still unexplained. Egen *et al.* (6) reported that there is still no definitive evidence that PLD and SH activity are one. The SH activity can be used as a predictive assay for the production of PLD by *C. pseudotuberculosis*, but it is inadequate technique to detect the actual concentration of PLD in culture supernatants which can be achieved by SDS-PAGE and immunoblotting technique using highly specific anti-PLD antibodies to maintain a proper amount of PLD antigen in each dose of toxoid vaccine.

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