

Full Length Research Paper

Evaluation of the synergistic haemolytic activity of phospholipase D produced by *Corynebacterium pseudotuberculosis*

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Accepted 27 November, 2012

Phospholipase D (PLD) is the major virulence factor in *Corynebacterium pseudotuberculosis* that exhibit synergistic haemolysis (SH) of sheep blood cells in the presence of products from *Rhodococcus equi*. 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). Fourteen isolates of *C. pseudotuberculosis* isolated from sheep with CLA and buffaloes with OSD "biotype 2" were identified by standard microbiological techniques and by multiplex PCR assay for direct detection of 16S rRNA gene, *rpoB* gene and *pld* gene specific for identification of *C. pseudotuberculosis* isolates. SH titers of all isolates were assayed by plate technique. The presences of PLD gene in supernatants of all isolates were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis immunoblot technique. The concentration of PLD gene was assayed by scanning the bound PLD gene with specific antibodies that appeared at 31.5 kDa. Results presented that multiplex PCR is rapid and specific for detection of *C. pseudotuberculosis* isolates and there is no correlation between titer of SH activity and the actual PLD genes concentration in culture supernatants. Moreover, the SH activity of PLD genes produced by biotype 2 was generally higher than those by biotype 1.

Key words: *Corynebacterium pseudotuberculosis*, OSD, CLA, PLD, multiplex PCR, synergistic haemolysis.

INTRODUCTION

Phospholipase D (PLD) is a potent exotoxin produced by *Corynebacterium pseudotuberculosis* of sheep origin (Hodgson et al., 1990; Lipsky et al., 1982; Tashjian and Campbell, 1995) and by buffalo isolates (Ghoneim et al., 2001). PLD is a secreted exotoxin that possesses sphingomyelinase activity and has been shown to increase vascular permeability *in vivo* (Batey, 1986;

Yozwiak and Songer, 1993), exhibit synergistic haemolysis (SH) of sheep blood cells in the presence of products from *Rhodococcus equi* and reduce the viability of ovine neutrophils (Batey et al., 1986). 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 (Egen et al., 1989; Paton et al., 2003; Piontkowski et al., 1998; Williamson, 2001). But field application of these toxoid vaccines showed controversy character while Eggleton et

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al. (1991) were satisfactory about protective efficacy of PLD toxoid vaccines. Other researchers reported that toxoid vaccines and inactivated corynebacterial cells (bacterins) provide partial protection (Brogden et al., 1996).

The reason of inadequate toxoid vaccines is still obscure, although Paton et al. (1995) 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 toxoid 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. The increase in SH activity is considered correlated to the concentration of PLD as included in culture supernatants, although there is still no definitive evidence that PLD and SH activity are one and the same (Egen et al., 1989). 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). Moreover, application of multiplex PCR reported by Pacheco et al. (2007) for rapid detection and identification of *C. pseudotuberculosis*.

MATERIALS AND METHODS

Clinical samples

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) (Eggleton, 1991, Zhao et al., 1991). Synergistic haemolysis with *Rhodococcus equi* ATCC 33701 and inhibition of β -haemolysis by *Staphylococcus aureus* ATCC 25923 were also evaluated (Eggleton, 1991, Zhao et al., 1991).

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 (Zhao et al., 1991). Biotypes were determined by conventional tests as described previously (Barakat et al., 1984) in addition to starch hydrolysis.

Starch agar hydrolysis test

It was 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 h at 37°C. The surfaces of inoculated media were flooded with Gram's iodine. Starch hydrolysis was indicated by the development of a clear zone around the colonies against a dark blue back ground.

Bacterial cultures. DNA isolation

Two different protocols were adapted for extracting DNA from pure bacterial cultures and clinical samples. Chromosomal DNA extraction from bacterial strains was carried out according to the standard protocol of Sambrook et al. (1989) 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 μ L 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 and 25 μ L 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 (Cetinkaya et al., 2002; McNamara et al., 1994; Pacheco et al., 2007). 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. Multiplex PCR were carried out according to Pacheco et al. (2007).

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 (Ghoneim et al., 2001). Three Boscat rabbits weighing 2.5 kg were inoculated subcutaneously with 1 ml dose containing 25 μ g rPLD protein mixed with complete Freund's adjuvant (Sigma) followed by 2 doses of 25 μ g rPLD mixed with incomplete Freund's adjuvant (Sigma) at weekly intervals. One week post last dose antibodies

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 |
| <i>pld</i> gene | 7/7 | 7/7 |

were assayed by ELISA (Simmons et al., 1998) 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.

Selective media for obtaining maximum yield 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 h, 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 h at 37°C.

Titration of synergistic haemolytic activity of culture filtrates according to Tachedjian et al. (1995)

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

Electrophoresis and immunoblotting

Total proteins in each culture filtrate were measured by Lowry et al. (1951) 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 min to ensure protein denaturation. Electrophoresis was performed (Laemmli, 1970). Briefly 10 µL of each treated concentrate were loaded into each lane and electrophoresis was done for 4 to 6 h 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 h, followed by

destaining of the gels till the background become completely clear. Proteins in some unstained gels were electrotransferred to nitrocellulose membranes (Towbin, 1979). Membranes that were blocked with blocking buffer (5% bovine serum albumin in 0.3% PBS-Tween, pH 7.2) for 2 h 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. (2001). The rPLD antigen was used for preparation of highly specific hyperimmune serum against PLD. This rabbit hyperimmune serum was used for binding to specific electrophoretic bands resulted in SDS-PAGE immunoblot technique

The strips were exposed to diluted rabbit hyperimmune serum (1:50) and incubated for 1 h at 25°C. The strips were washed 3 times (5 min 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/H₂O₂ (0.5 mg /ml/0.15% in PBS with 17% methanol) as substrate. Inoculation with substrate was in dark at 25°C for 45 min.

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 (Figure 1) can distinguish these organisms.

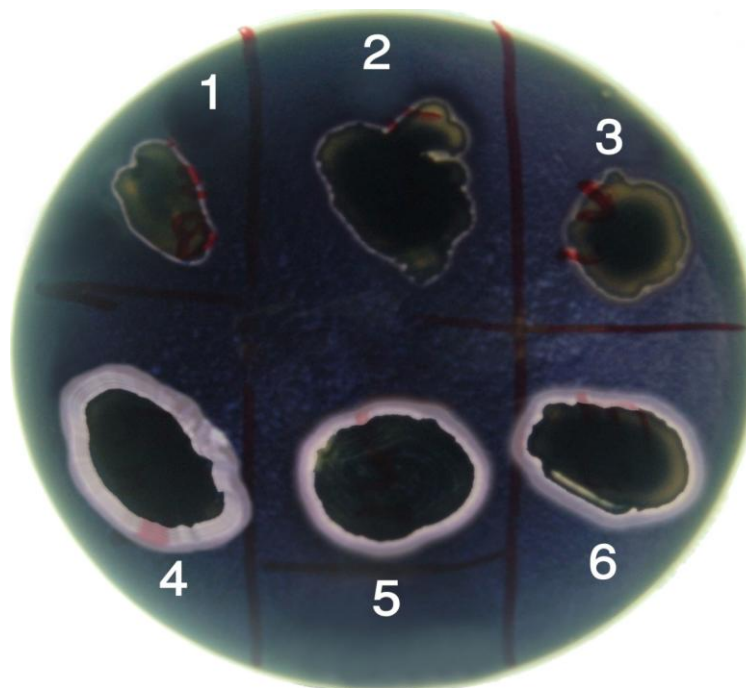


Figure 1. Starch hydrolysis plate showing clear zone of hydrolysis around colonies of *C. pseudotuberculosis* of buffalo origin Number. (4, 5, 6) and sheep isolates Number (1, 2, 3) showing no hydrolysis.

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 shows 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.

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

DISCUSSION

In order to improve *C. pseudotuberculosis* detection by PCR, some authors recently designed a multiplex PCR (mPCR) assay and adapted protocol to extract bacterial genomic DNA directly from clinical samples. Amplification of multiple loci in a single reaction through mPCR is currently a powerful and widely used tool for the rapid and specific identification of pathogenic bacteria (Çetinkaya et al., 2002; Dorella et al., 2006; Paccheo et al., 2007). Multiplex PCR targeting three *C. pseudotuberculosis* genes: (a) the 16S rRNA gene, which is the gene of choice for most microbial taxonomy studies (Çetinkaya et al., 2002; Khamis et al., 2005); (b) the RNA polymerase β -subunit gene (*rpoB* gene), which is currently used for the study of phylogenetic relationships in the genera *Corynebacterium* and *Mycobacterium* (Dorella et al., 2006); and (c) genes encoding exotoxin PLD (*pld*), which is a sphingomyelinase implicated in the virulence of *C. pseudotuberculosis*, *C. ulcerans* and *Arcanobacterium haemolyticum* (McNamara et al., 1995). Therefore, this technique enabled specific identification of *C. pseudotuberculosis* isolates in culture and direct detection in pus samples from local CLA-affected

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 titer | Concentration of PLD in $\mu\text{g/ml}$ |
|--------------------|----------------|------------------|--|
| 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 |

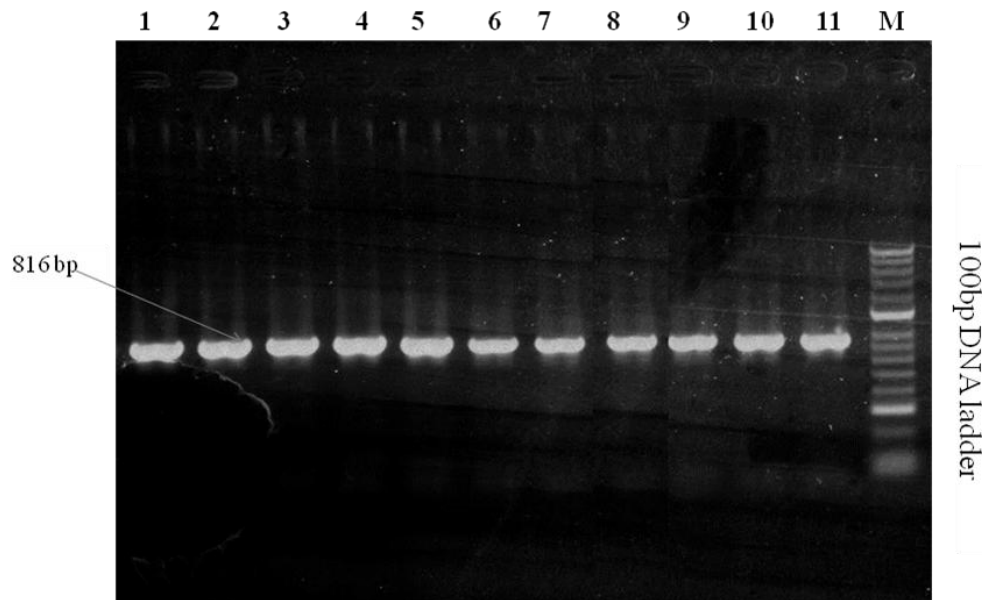


Figure 2. PCR amplification of 16s rRNA 816-bp DNA product from sheep and buffalo isolates of *C. pseudotuberculosis*.

animals. Therefore, all the strains were tested by PCR and multiplex PCR reported by Paccheo et al., (2007) to confirm that the strains belonging to *C. pseudotuberculosis* as shown in Figures 2, 3 and 4.

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

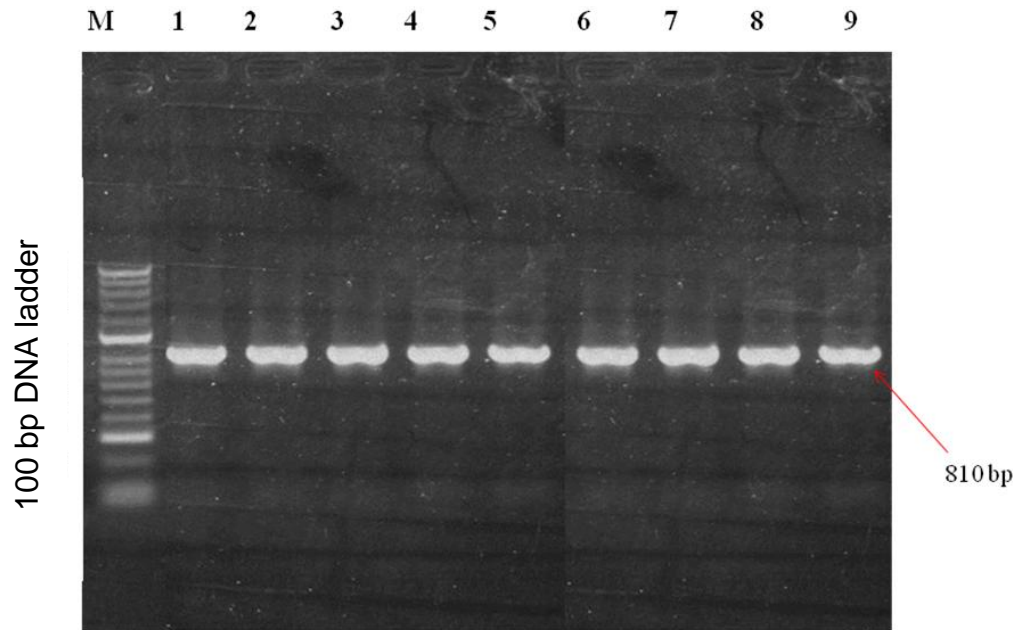


Figure 3. Agar gel electrophoresis showing amplification product of phospholipase D gene from 4 sheep isolates No (1,2,3,4) and 4 buffalo isolates number 5,6,7,8 of *C. pseudotuberculosis* showing 910 bp bands. Lane 9 positive control.

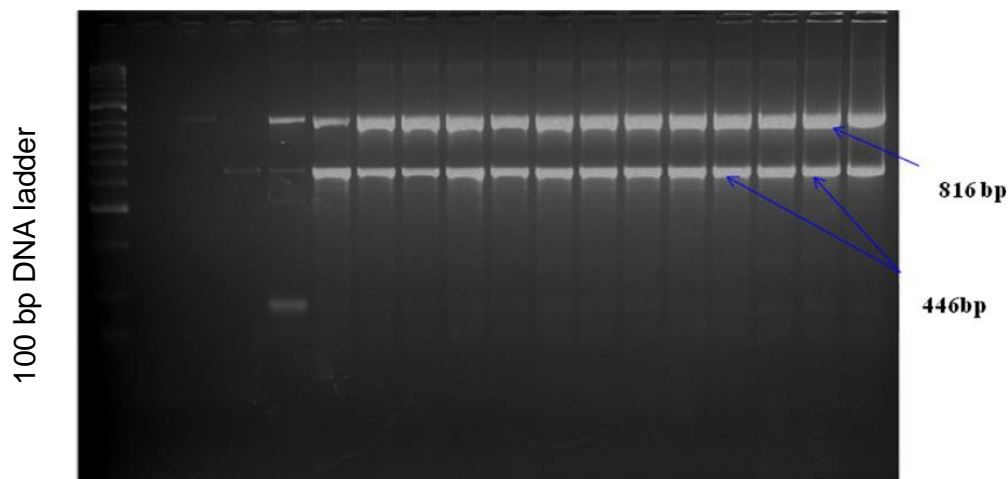


Figure 4. Agarose gel electrophoresis showing multiplex PCR amplification of 446 bp fragments of *rpoB* gene and 816 base pair fragments of 16S rRNA of *C. pseudotuberculosis*.

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 its 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 (Egen et al., 1989). 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 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. (Egen et al., 1989) 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 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 and 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, (1983) and Muckle and Gyles (1986) reported a poor correlation between PLD enzyme contents in supernatants of *C. pseudotuberculosis* measured by radiometric assay. Egen et al. (1989) reported that isolates having 3 fold variations in PLD concentration measured by radiometric assay produced nearly identical areas of haemolysis on *Rhodococcus* 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. (1989) 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 an 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.

ACKNOWLEDGEMENT

"The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding of the work through the research group project No.: RGP-VPP-162."

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