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ORIGINAL ARTICLE

Immunological characterization of diphtheria toxin recovered from *Corynebacterium pseudotuberculosis*

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Abstract Diphtheria toxin (DT) is a potent toxin produced by the so-called diphtheria group which includes *Corynebacterium diphtheriae* (*C. diphtheriae*), *Corynebacterium ulcerans* (*C. ulcerans*), and *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*). The present investigation is aimed to study in detail the production of DT by *C. pseudotuberculosis*. Twenty isolates were obtained from sheep diseased with caseous lymphadenitis (CLA) and twenty-six isolates were obtained from 26 buffaloes diseased with oedematous skin disease (OSD). All isolates were identified by standard microbiological and DT production was assayed serologically by modified Elek test and immunoblotting. All sheep isolates were nitrate negative, failed to hydrolyze starch and could not produce DT, while all buffalo isolates (biotype II) revealed positive results and a specific band of 62 kDa, specific to DT, was resulted in all concentrated cell fractions (CF), but was absent from non-toxigenic biotype I isolates. At the same time, another band of 31 kDa specific to the PLD gene was obtained with all isolates of biotype I and II. Moreover, all isolates showed positive synergistic hemolytic activity and antagonistic hemolysis with β-hemolytic *Staphylococci*. The obtained results also indicated that *C. pseudotuberculosis* could be classified into two strains; non-toxigenic biotype I strain, which failed to produce DT as well as being negative to nitrate

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and starch hydrolysis, and toxigenic biotype II strain, which can reduce nitrate, hydrolyze starch as well as produce DT.

1. Introduction

Corynebacterium pseudotuberculosis is the causative organism of a chronic disease in sheep and goat known as caseous lymphadenitis (Fontaine et al., 2006). In horse, it causes external abscesses as well in internal organs or limb infection in the form of ulcerative lymphangitis (Foley et al., 2004). In Egypt, it causes a buffalo disease known as oedematous skin disease which is characterized by redness and swelling at the site of infection which almost initiated in hairless areas of the skin (Moussa et al., 2014). Swelling may extend to drainage of lymph nodes and may involve the whole hind or fore limbs (Barakat et al., 1984). It also causes sporadic cases in other species including human and cattle (Mills et al., 1997; Peel et al., 1997). Two biotypes and serotypes of *C. pseudotuberculosis* have been identified on the basis of difference in nitrate reduction, guinea pigs inoculation, and antigenic structure (Barakat et al., 1984; Baird and Fontaine, 2007; Baird and Malone, 2010). In contrast isolates from sheep and goats, *C. pseudotuberculosis* isolated from buffaloes can reduce nitrate to nitrite, while isolates from cattle may reduce nitrate and may not. On the other hand, natural cross species infection does not commonly occurr (Foley et al., 2004). The major virulence factor in *C. pseudotuberculosis* is the exotoxin phospholipase D (PLD) (McNamara et al., 1995). Although *C. pseudotuberculosis* is considered one of diphtheria group members, toxigenic strains of these bacteria can produce diphtheria toxins (Kraeva et al., 2007). DT toxin is a potent toxin composed of a single polypeptide chain with molecular weight of 62 Kilodalton (kDa). It contains two fragments, A and B, and both are required for intoxication of tissue culture cells or animals. Fragment A has the active site of DT and is responsible for the enzymatic activity of DT, while fragment B is responsible for the attachment of DT with receptors on host cells. *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, and *C. pseudotuberculosis* are converted to DT producers after their infection with the ß-corynebacteriophage encoding the diphtheria toxgene. DT-producing *C. diphtheriae* and *C. ulcerans* were deeply investigated and characterized (Seto et al., 2008), while DT production by *C. pseudotuberculosis* is not well documented however, Maximescu et al. (1974) and Stanford et al. (1998) reported the production of DT by two isolated strains from Egyptian buffaloes. Therefore, the purpose of the current study was to investigate DT production by *C. pseudotuberculosis* biotypes I and II that were isolated from sheep and buffalo, respectively. Moreover, phenotypic characterization was done of all isolates recovered from buffaloes and sheep.

2. Materials and methods

2.1. Bacterial isolates and sera

The study was undertaken with 46 clinical isolates of pathogenic *C. pseudotuberculosis*, 20 isolates out of the total 46 were obtained from clinically CLA-infected sheep, while the other 26 isolates obtained from buffaloes showed OSD typical symptoms. *C. diphtheriae*, Park Williams 8 (PW8) strain, was used as the reference strain of this study. Standard anti-DT hyperimmune serum was purchased commercially (Egyptian Holding Company for Biological Products and Vaccines; VACCERA, Giza, Egypt), while all DT-positive sera were collected from OSD-infected buffaloes.

2.2. Standard microbiological and genotyping of isolates

Biotyping of isolates was performed using API Coryne (BioMerieux, France) according to the manufacturer's instructions. All isolates were identified for starch hydrolysis activity (Moussa et al., 2014). Briefly, brain heart infusion agar mixed with 0.4% starch was spot-inoculated with tested bacteria. Plates were incubated at 37 °C for 48 h, and then were covered with gram's iodine. Iodine reacts with starch to form a dark blue background and clear areas around the spots of inoculum will appear. The total DNA of all strains were isolated as described previously by Pallen et al. (1994) and used for the identification of 16s rRNA and PLD genetic characteristics of *C. pseudotuberculosis*. The detection of 16s rRNA gene of different isolates was carried out using PCR with purified DNA and the primer sequence of Dorella et al. (2006). Additionally, all isolates were examined for the PLD gene using PCR technology according to Moussa et al. (2014).

2.3. Detection of DT using Elek's test

All isolates were screened by modified Elek's immunoprecipitation test; also known as modified Antitoxin in Well (AIW), according to Pimenta et al. (2008). Briefly, Elek's-based agar media supplemented with newly-born bovine serum at a ratio of 1:5 (v/v) were poured in 9 ml Elek's agar plates. After solidification, a central hole of 5 mm diameter was made using a sterile stainless steel borer. The well was filled with 9 µl of standard anti-DT serum. In other plates, the wells were filled with positive sera collected from diseased buffaloes with OSD. Central hole was surrounded by a loopful of individual isolates which were streaked and stabbed into agar at a distance of 10 mm from the edge of the central well. *C. diphtheriae* PW8 (DT-producer strain) was used a positive control in each set of wells.

2.4. Detection of DT by immunoblotting

All isolates were grown on brain heart broth medium for 48 h at 37 °C; supernatants were collected by centrifugation at 4000 rpm for 10 min, filtered through bacteriological filters (45 µm). The filtrates were concentrated to 1/20 of the original volume; concentrated filtrates were treated with sodium dodecyl sulfate (SDS) and reducing agent β-mercaptoethanol, and

then separated by SDS–poly acrylamide gel electrophoresis (SDS–PAGE) in 12.5% acrylamide gel. Standard DT was commercially purchased (VACCERA, Egypt) and included on acrylamide gels to confirm the location of toxin protein. Two sets of gels were immunoblotted on nitrocellulose membranes and one set of blotted membranes was treated with standard anti-DT serum, and developed with alkaline phosphatase-labeled anti-horse IgG antibodies to detect the fractionated proteins. The other set was treated with serum collected from diseased buffaloes with OSD and developed with alkaline phosphatase-labeled anti-bovine IgG antibodies (Sigma, USA). Substrates containing nitroblue tetrazolium (100 μg/ml) and 5-bromo-4-chloro Indolyl phosphate (50 μg/ml) were incubated with blotted membranes to bind the Fc-region of labeled antibodies. Sera were defined as western blot "Positive" when a clear reaction is developed within 10 min across most of the molecular mass spectrum of antigens present on the membranes.

3. Results

3.1. Bacteriological and biochemical identification

All isolates of sheep and buffalo origin (biotype I, and II respectively) showed the same trend of reaction with some individual variation in fermenting sugars. All isolates resulted in positive synergistic hemolytic activity and antagonistic hemolysis with β-staphylococcus hemolytic activity. On the other hand, both biotypes were differentiated by nitrate reduction test and starch hydrolysis activity. All sheep isolates (biotype I) were negative for both assays, while isolates of buffalo (biotype II) could reduce nitrate and hydrolyze starch as shown in Fig. 1.

3.2. Identification of isolates by PCR assay of 16s rRNA and PLD genes

Both biotypes I and II isolates of *C. pseudotuberculosis* yielded a similar PCR profile of 816 bp and 910 bp amplicons corresponding to 16s rRNA and PLD genes, respectively.

3.3. Detection of DT produced by C. pseudotuberculosis

All isolates (biotype I and biotype II) were assayed by modified Elek's test (IW assay). Biotype I isolates failed to develop precipitation lines against the central well that contained standard anti-DT serum, while biotype II isolates developed clear and reproducible bands of identity with PW8 strain of *C. diphtheria* (Fig. 2). In the case of using positive control serum obtained from buffaloes diseased with OSD, lines of precipitation bands were developed adjacent to the control well forming an incomplete ring of lines of identity with the biotype I and biotype II, while PW8 failed to develop this line of precipitation. At the same time, biotype II isolates (toxigenic) developed another precipitation line of identity with PW8 diphtherial strain (DT producer), while biotype I failed to develop this line as shown in Fig. 2.

3.4. Immunoblotting detection of DT by C. pseudotuberculosis

Concentrated cell fractions (CF) of both biotypes were electrophoresed by SDS–PAGE and protein blots were transferred onto nitrocellulose membranes. Using these membranes, two types of positive sera were used for reacting with blots. The first hyperimmune serum was obtained commercially (VACSERA, Giza, Egypt) and used as standard containing anti-DT antibodies, while the second was positive serum collected

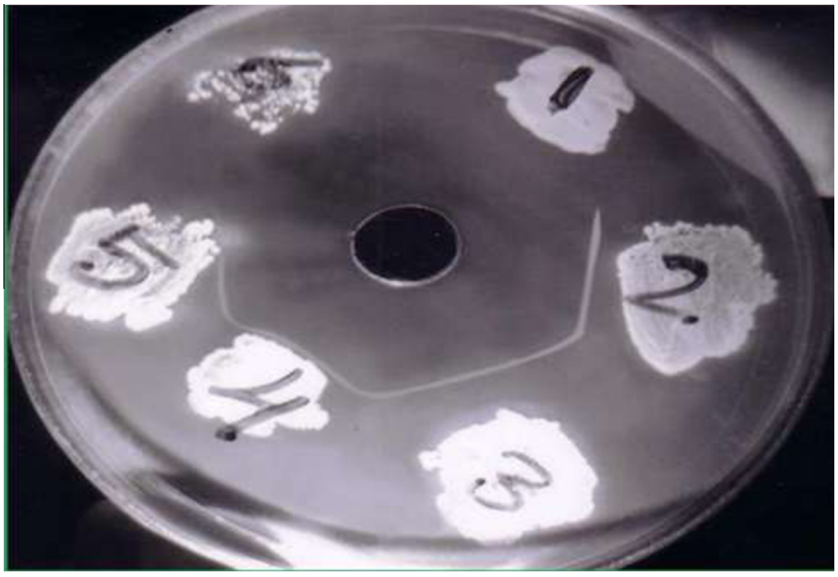

Figure 1 AIW test with a 5 mm diameter center well containing 9 μl of anti DT surrounded by 6 different *Corynebacteria*. Streaks 1 and 6 are sheep isolates, streak 2 is standard PW8 *C. diphtheriae* and streaks 3, 4 and 5 are buffalo isolates showing lines of identity with the PW8 strain. Sheep isolates (1 and 6) failed to produce DT toxins.

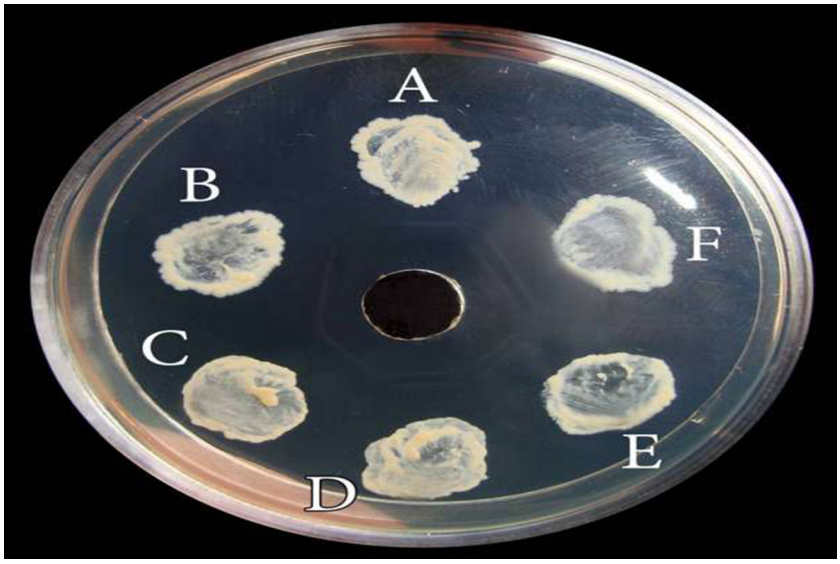

Figure 2 Elek plate with a central well contains positive serum collected from diseased buffaloes with OSD. Streak A is PW8 and streaks F, E and D are buffalo isolates showing an outer line of identity with PW8 producing DT. Streaks F, E, D, C and B show inner lines due to the production of PLD by all isolates. No inner line was seen with PW8.

Figure 3 Immunoblotting of concentrated culture filtrates from 4 sheep isolates (lane 2–5) and of 9 buffalo isolates in lane (6–14) and lane 1 contain standard anti-DT serum and lane 15 a broad range protein marker. Bands were detected by positive serum obtained from buffaloes with OSD. All buffalo isolates show a band at 62.0 KDa at the specific region of standard anti DT. Know DT bands appeared with sheep isolates. All sheep and buffalo isolates develop a band at 31 KDa at the specific region of PLD protein.

from buffaloes diseased with OSD. A specific band of 62 kDa, specific to DT, resulted in all concentrated cell fractions (CF) of biotype II isolates in addition to the positive DT control obtained commercially, but was absent from non-toxigenic biotype I isolates. At the same time, another band of 31 kDa that was specific to the PLD gene was obtained with all isolates of biotype I and II. The 31 kDa band was not developed with PW8 diphtherial strain as shown in Fig. 3.

4. Discussion

The acute toxigenic nature of *C. pseudotuberculosis* isolated from swellings of diseased buffaloes with OSD (Selim, 2001; Pratt et al., 2005), and the high killing effect of isolates inoculated into guinea pigs, indicate the production of exotoxin, other than PLD, by all buffalo isolates (biotype II) and ovine isolates (biotype I). It is well documented that *C. pseudotuberculosis* of ovine origin (biotype I) induce a chronic disease in sheep and goats known as caseous lymph adenitis (CLA), and when inoculated in guinea pigs they produce abscesses at the inoculation site and death may occur within 15 days (Barakat et al., 1984; Stanford et al., 1998). The major virulence factor in both non-toxigenic biotype I and toxigenic biotype II of *C. pseudotuberculosis* is PLD, which cannot be incriminated in the induction of an acute disease like OSD in buffaloes. One of the characteristic manifestations of OSD is

the development of deep and large ulcers that may be associated with sloughing of large areas of the skin (Selim, 1999, 2001). Ulcer formation is one of the featured lesions of diseases caused by *C. diphtheriae* and *C. ulcerans*. Both species produce the diphtheria toxin, while *C. ulcerans* produce PLD additionally. Production of DT by *C. pseudotuberculosis* is obscure since the publications of Maximescu et al. (1974); they reported the production of DT by two strains of *C. pseudotuberculosis* isolated from two different Egyptian buffaloes. To confirm these data, we isolated 20 isolates from different sheep infected with CLA and 26 isolates from OSD-diseased buffaloes. Biochemical identification of all isolates revealed their similarity in sugar fermentation, with the presence of individual variation. But all isolates were identified in some assays as synergistic hemolytic activity and inhibition of B-staphylococcal hemolytic activity, presence of 16s rRNA and PLD genes. PLD gene is a constant marker which can differentiate *C. pseudotuberculosis* and *C. ulcerans* from *C. diphtheria* (Mills et al., 1997; Pacheco et al., 2007). On the other hand, *C. pseudotuberculosis* biotypes I and II can be differentiated by the presence of two criteria; the first criteria is the nitrate reduction test where biotype II of *C. pseudotuberculosis* reduce nitrates, and this result goes in hand with previous studies in the same context (Songer et al., 1988). The second criteria is starch hydrolysis feature which can differentiate between biotypes I and II, the latter can hydrolyze starch while biotype I fails to perform such hydrolysis (Fig. 3). Production of DT by *C. pseudotuberculosis* either of biotype I or II was assayed with modified Elek precipitation for the detection of DT production. Two types of positive anti-sera containing anti-DT antibodies were used in the reaction. Biotype II isolates of buffaloes developed identical lines of precipitation like those produced by PW8 DT-producing *C. diphtheriae*, while the ovine biotype I isolate failed to develop any precipitation lines. Another profile of precipitation lines were developed when positive anti-sera obtained from diseased buffaloes were used. Two precipitation lines appeared; the first line appeared adjacent to the central well and was developed by all isolates of biotype I and II, while the second line of precipitation was outer to the first line. Interestingly, the first line of precipitation was not developed by DT-producing PW8 diphtherial strain. The absence of the antigen produced by *C. pseudotuberculosis* biotypes I and II was seen. This antigen may be the PLD which is produced by both biotypes I and II and is a characteristic feature of *C. pseudotuberculosis*. This confirms data obtained by PCR which revealed the presence of the PLD gene in DNA of both biotypes of *C. pseudotuberculosis*. These lines of precipitation were developed between the biotype II of buffalo isolates and the positive control of DT-producing strain PW8. These lines of identity indicate the production of DT by *C. pseudotuberculosis* biotype II, while isolates of biotype I could not develop these lines of precipitation. These results indicate that sera of OSD-diseased buffaloes contain antibodies against PLD and DT antigens. At the same time, *C. pseudotuberculosis* of biotype II produce PLD and DT exotoxins, while biotype I isolates produce only PLD. The results confirmed data reported by Maximescu et al. (1974), they found that two isolates of *C. pseudotuberculosis* obtained from Egyptian buffaloes were toxigenic, while all ovine isolates (biotype I) failed to produce DT. It is well established that DT-production character is accepted after integration of β-corynebacteriophage into non-toxigenic strains of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* (Wong and Groman, 1984). Although biotypes I and II were integrated by corynebacteriophage, yet biotype I (non-toxigenic strains) could not produce DT, while biotype II isolates were toxigenic. It can be concluded that *C. pseudotuberculosis* can be typed into two types; DT-toxigenic and non-toxigenic types. Toxigenic types are characterized by their activity to reduce nitrates, hydrolyze starch, while non-toxigenic are nitrate negative, not able to hydrolyze starch.

5. Conclusion

The obtained results also indicated that *C. pseudotuberculosis* could be classified into two strains; non-toxigenic biotype I strain, which failed to produce DT as well being negative to nitrate and starch hydrolysis, and toxigenic biotype II strain, which can reduce nitrate, hydrolyze starch as well as produce DT.

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