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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