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Cytotoxic Activity in Fermenter Culture Supernatants of *Corynebacterium pseudotuberculosis*

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Abstract: Fermenter Culture supernatants of *Corynebacterium pseudotuberculosis* strain 3450 NCTC grown in a continuous culture were tested for cytotoxicity in 7 cell lines. The supernatants were toxic to EBL, MDCK, NBL, CHO-K1, 3T3, BHK and Vero cells. The cytotoxic activity was used as an assay method to determine the optimal incubation temperature and pH for cytotoxin production in fermenter. Fermenter culture supernatants of a reference strain *C. pseudotuberculosis* and three field isolates were compared for their cytotoxicity to BHK cells. Two of the field isolates were non-cytotoxic to BHK cells, one isolate had weak cytotoxicity and the reference strain was the most cytotoxic. SDS-PAGE of fermenter culture supernatants of the 4 strains revealed 35 to 39 bands with molecular weights ranging from 24 to 149 KDa.

Key words: *Corynebacterium pseudotuberculosis*, fermenter, cytotoxicity, cell lines

INTRODUCTION

Corynebacterium (C) *pseudotuberculosis* is the causative agent of caseous lymphadenitis (CLA) in sheep and goats, ulcerative lymphangitis and the syndrome of c hronic ventral abscesses in horses. The organism has been isolated from abscesses and other suppurative processes in a variety of animal species and man[1]. It produces an exotoxin that has been shown to play a role in the pathogenesis of CLA[2]. The exotoxin of *C. pseudotuberculosis* has been used in the context of a single protein with phospholipase-D (PLD)[2], dermonecrotic and lethal[3], *staphylococous* beta-hemolysin inhibitory[4] and synergic hemolysis with *Rhodococcus equi* factors[5].

The PLD has been found in all isolates of *C. pseudotuberculosis* tested to date[6]. Nevertheless, it is still unclear whether the crude exotoxin consists of several toxin-proteins or contains only one protein of higher molecular weight that decomposes to proteins of lower molecular weight that retain the immunoreactive epitopes.

The objectives of this study were to determine the presence of cytotoxic activity in fermenter culture supernatants of *C. pseudotuberculosis* grown in a continuous system. Pertinent matters such as the effect of applying different fermentation temperatures and pH values on the cytotoxin production and SDS-PAGE analysis of the supernatants were also studied.

MATERIALS AND METHODS

Bacteria: *C. pseudotuberculosis* strain NCTC 3450, isolate 24 (natural goat strain), isolate 226 (natural sheep isolate) and isolate 26 (natural sheep isolate).

Cell lines: EBL, MDCK, NBL, CHO-K1, 3T3, BHK and Vero cell lines were used in the study. Cells were grown for 4 days in tissue culture flasks with Eagle's modified essential medium supplemented with 5-10% fetal bovine serum at 37°C in 5% CO_2 in a cell incubator (HERAEUS). A cell suspension containing 1.0x 10^5 cell/ mL was used for cytotoxicity testing.

Cytotoxicity: A single fermenter sample of the reference strain grown at optimal fermentation parameters was tested 12 times in each of the 7 cell lines. Samples taken at different fermentation temperatures and pH were tested in BHK cells to determine the effect of these physical factors on the cytotoxin production. Fermenter samples of the four strains grown under the same optimal physical parameters were used to compare their cytotoxicity to BHK cell line.

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The test was conducted in tissue culture 96-well plates (SARSREDT). Samples were adjusted to pH 7.4, centrifuged at 6720 g 10 min^{-1} and 50 µL of each sample was two fold diluted in duplicate in cell medium containing 100 I.E. mL penicillin and 100 µg mL^{-1} streptomycin, 50 µL cell medium was added to each dilution and finally 50 µL of cell suspension was added. Controls of bacterial growth medium, undiluted toxin and the cell medium were included in each plate. The preparation was incubated at 37 °C under 5% CO_2 for 72 h in a cell incubator.

A modified MTT staining procedure from the method described by Barer *et al*.[7] was used. After incubation 20 µL MTT (5 mg mL^{-1}) solution was added to each well and incubated for 4 h at 37 °C. The living cells will be selectively stained with MTT and become deposited to the bottom of the wells, supernatant MTT was then discarded and 120 µL isopropanol-HCL-SDS solution (990 mL isopropanol+10 mL HCL+3% SDS) was added to each well, incubated for ½ h, mixed well and the extinction was read at 750 nm in an ELISA reader (ASYS HITECH) connected to a computer (DigiWin, Mikrotek).

Calculation of the 50% cytotoxicity titre was performed according to the MTT-test principal[8]. The highest dilution of the toxin that causes 50 % death of the cells is defined as the activity of the sample in cytotoxic units per mL (CU mL^{-1}).

SDS-PAGE of fermenter supernatnats: 6 µL fermenter supernatants of the four strains were solubilized in sample buffer (0.125 M Tris base, 4.0 % SDS, 20 % glycerol, 2 mM EDTA, Bromophenol blue 0.02 %) and separated by electrophoresis on 10-12 % SDS-PAGE gradient gel prepared from BIO-RAD 30 % acrylamide/Bis solution. BIO-RAD SDS-PAGE standards were used as molecular weight markers. Samples were electrophoresed (BIO-RAD POWER/ PAC 3000) (100 watt 5 min^{-1}, then 200 watt 40 min^{-1} and finally 100 watt 5 min^{-1}) and proteins were visualized by staining with BIO-RAD silver stain protocol in automated gel stainer (Pharmacia Biotech). Stained gels were photographed (MWG- BIOTECH system, software, Gel Print 2000I, Bio Photonics) and the molecular weights of separated proteins were resolved with RELPscan Plus 3.0. This program was also used to determine the total number of bands in each lane, shared bands, the distance and the similarity index.

RESULTS

Cytotoxicity: Supernatants of *C. pseudotuberculosis* strain NCTC 3450 were cytotoxic to the seven cell lines; however there were quantative differences. Vero cells were the most sensitive (1018 CU mL^{-1}) to the cytotoxic affect and CHO cells were the least sensitive (65 CU mL^{-1}).

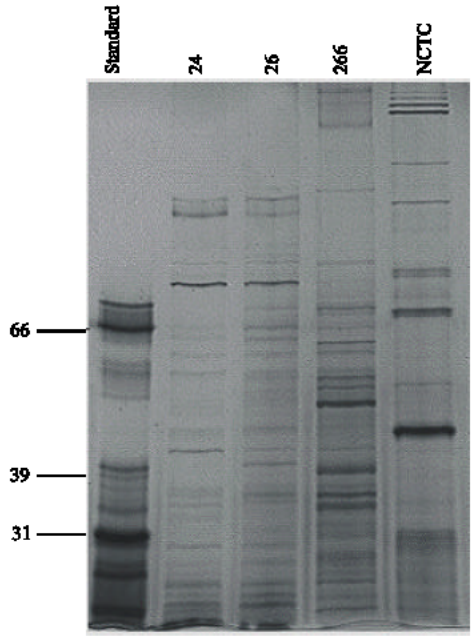

Fig. 1: SDS-PAGE profile of four *C. pseudotuberculosis* strains

Fermenter samples obtained during the optimization of the physical parameters for cytotoxin production were tested for their cytotoxicity to BHK cell line. The highest cytotoxicity was obtained at 31 °C and pH 8.0 (299 CU mL^{-1}).

Comparing the cytotoxic activity of the 4 strains to BHK cells, demonstrated that the culture supernatants of strains NCTC and 226 were cytotoxic while strains 24 and 26 were noncytotoxic.

SDS-PAGE: Fermenter samples of the four strains analysed by SDS-PAGE and stained with the silver protocol were very complex and demonstrated between 35 to 39 bands. The highest similarity index was 0.68 between strain 24 and 26 and the least was between the reference strain and 26 Fig. 1.

DISCUSSION

Fermenter supernatants of *C. pseudotuberculosis* demonstrated the presence of cytotoxic activity as an additional activity of the exotoxin. The cytotoxicity was not only limited to one cell line but to all cell lines tested. The highest cytotoxicity was demonstrated in Vero cells and the least was detected in CHO cell line. No reports

were found in the available literature describing the cytotoxic activity of the *C. pseudotuberculosis* exotoxin, but the PLD, which the only identified molecule in the crude exotoxin had been shown to be active against sphingomyelin in the membranes of endothelial cells[9] and erythrocytes[5,10] and was also found to affect the function of normal ovine neutrophils[11].

The toxicity of *C. pseudotuberculosis* to murine and caprine macrophages was attributed to the action of the toxin through its sphingomyelinase activity. Intracellulary the organism liberated sufficient amounts of cytotoxin to cause membrane damage and eventual lysis of the macrophages[12,13]. The cytotoxicity of *C. pseudotuberculosis* exotoxin reported here might probably be expressed in a similar manner by the direct action of the exotoxin on the cytoplamic membranes of the cell lines.

The optimized physical conditions for growth in the fermenter enhanced exotoxin production, since the cytotoxicity could not be demonstrated in supernatants obtained from cultures in test tubes (unpublished data).

The cytotoxicity detected in the fermenter supernatants was utilized as test to determine the cytotoxic potentiality of the fermenter samples taken during the optimization of temperature and pH associated with the best levels of toxin production. Although Vero cells were the most sensitive to the exotoxin's effect, BHK cell line was chosen in the assay since they are rapid growing compared to Vero.

The comparative study of the strains revealed that strains 226 and NCTC 3450, both have cytotoxic activity, when injected subcutaneously in mice failed to cause abscesses at the inoculation site, whereas strains 24 and 26, both were completely non-cytotoxic to BHK cells, when injected in mice caused the development of abscesses at injection sites (unpublished data). These findings indicated that pyogenic strains are non-cytotoxic, while the cytotoxic strains are non-pyogenic. The fact that the strains differed in cytotoxiciy and pyogenicity could be utilized as a tool to type *C. pseudotuberculosis* strains since there is no means to type the strains of this species except for the biotyping according to the nitrate reducing ability[14].

There are conflicting reports in literature about the extact molecular weight of the exotoxin; in this study SDS-PAGE of *C. pseudotuberculosis* fermenter supernatants demonstrated numerous bands. The controversy in the molecular weight masses reported in literature may be partially attributed to the different types of media used for preparation of the exotoxin and to the methods followed to analyse the exotoxin. SDS-PAGE profile of *C. pseoudotuberculosis* strains was dissimilar and accordingly different molecular weights might be predicted from these profiles, this fact may have additionally created the reported controversy.

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