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Semiquantification of lethal toxin at the bottom of reactors for *Clostridium sordellii* culture

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ABSTRACT

The production of *Clostridium sordelli* veterinary vaccine is prepared by culturing the microorganism in large industrial bioreactors and purifying the toxin from culture supernatants. Currently, the harvesting time is established as function of bacterial growth, but not always this moment is associated to the maximum concentration of toxin present in the supernatant. Thus, it is easy to find different total amounts of toxin produced batch to batch. Besides, at present the toxin concentration is measured by *in vivo* LD50 methods, and results can be obtained only after 72h, when the bioreactor was already stopped. Moreover, the method is labour intensive and requires the use of a significant number of experimental animals We describe the development of a latex agglutination reagent for semiquantification of *Clostridium sordelli* lethal toxin (TcsL) and its use in industrial bioreactors. The reagent was developed and characterized in our laboratory achieving a considerable low detection limit (8ng of toxin per ml of culture supernatant) and then it was validated in actual industrial conditions. The use of such a rapid (i.e, in minutes) and easy to use reagent will allow to follow the culture in real time and thus standardizing the optimal end-point for harvesting in terms of toxin quantity. As an immediate consequence, the efficiency of TcsL industrial production may be optimized.

KEYWORDS

TcsL;
Clostridium sordellii;
Latex agglutination tests;
Clostridial toxins;
Industrial cultures.

INTRODUCTION

C. sordellii is an anaerobic gram-positive bacillus that causes infections in humans and animals[1,2]. It is an important veterinary pathogen, causing gas gangrene[3], liver disease haemorrhage, ulcers, abomasal bloat, myositis, and sudden death in sheep[4-6]. Although human infections caused by this pathogen are relatively uncommon, *C. sordellii* has been associated with human maternal deaths, presenting with symptoms that are similar to those of toxic shock-like syndrome[7, 8].

The *C. sordellii* toxins include phospholipase C[9], DNAse[10], neuraminidase[11], haemolysin[12], and 2 extracellular toxins, that is, haemorrhagic toxin (TcsH) and lethal toxin (TcsL)[1,2,13]. The pathogenic *C. sordellii* strains produce either TcsL alone or both TcsL and

FULL PAPER

TcsH[14,15]. TcsL (MW, 240–250 kDa; pI, 4.55[13]is a member of the large clostridial toxin (LCT) group that catalyses glucosylation on various small G proteins[16,17,18]. It is described as a major virulence factor[18] and, like other LCTs, is composed of 3 domains that act sequentially during cell infection[19]. The C-terminal domain is involved in the specific recognition of cell surface receptors. Following endocytosis, the central hydrophobic domain penetrates the endosomal membrane, triggering mechanisms linked to the translocation of the N-terminal domain into the cytosol[20, 21]. The N-terminal domain, which is responsible for the glucosyltransferase activity, is then liberated into the cytosol by proteolytic cleavage and glucosylates intracellular targets[22].

Immunisation with the toxoids of TcsL and TcsH offers protection against *C. sordellii* infections[23], and these toxoids are useful for preparing vaccines for veterinary use. Both toxins are obtained industrially from *C. sordellii* culture supernatants. Because the concentrations of these products vary during culture processing, a control method is required for optimizing their yields. The method currently used to assay LcsT in culture medium involves determination of the lethal dose at 50% (LD50)[0]. This method is labour intensive, uses experimental animals, and data can be obtained only after 72 h. In this work, we report the development of a rapid semi-quantitative immunoassay for monitoring the real-time levels of TcsL in *C. sordellii* cultures. For that purpose, TcsL was purified from culture supernatants of C. Sordellii and used as immunogen to obtain specific anti-TcsL antibodies. Then latex particles were coated with the antibodies. This allowed the preparation of an immunoreagent, to use as an alternative strategy of toxin detection, directly on culture supernatant samples at the bottom of reactor. The use of direct agglutination of latex particles, offers several advantages, including ease of use, rapid semiquantitative analysis to determine peak yields, and being an *in vitro* assay, the elimination of the use of experimental animals.

MATERIALS AND METHODS

C. sordellii culture supernatant samples were supplied by Prondil SA (Uruguay).

Experimental animals use

The protocols for the use of experimental animals were approved by the Comisión Honoraria de Experimentación Animal, Universidad de la República.

Purification of LcsT from *C. sordellii* culture supernatant

Purification of LcsT was achieved by salting-out precipitation on supernatants of *C. Sordellii* cultures. Firstly, a clostridial culture was centrifuged at 10.000 rpm for 20 min at 4º (Beckman Avanti, USA), to separate cell debris; then and aliquots (200 ml) were used to analyze the best precipitation conditions in order to optimize yield and purity of the precipitated toxin. Salting-out precipitation was assayed at final concentration of 30%, 40%, and 50% (w/v) of total saturation of ammonium sulphate (Sigma, USA) [0]. The precipitates obtained for each condition were centrifuged, then dissolved in phosphate-buffered saline (PBS) and extensively dialyzed against PBS.

The protein concentration of dialyzed samples was estimated by BCA Protein Assay reagent (Pierce, Holland) and all fractions were evaluated by SDS-PAGE 8% (w/v) under reducing conditions[26]. Briefly, a 10 μg sample of each fraction was loaded in a gel lane, and electrophoresis was performed for 1 h at 25 mA, then the gel was stained with Coomassie Blue R-250 (Sigma, USA). To confirm the presence of the toxin, the band corresponding to greater than 220KDa was sliced from the gel, and submitted to mass spectrometry analysis (MS; MALDI-TOF/TOF 4800 Analyzer; Applied Biosystems, Framingham, USA).

Subsequently, 500 μg of the grater than 220KDa band -enriched fraction, was electrophoresed on a preparative SDS-PAGE 8% under reducing conditions and the gel was stained with zinc-imidazole[27]; then the band corresponding to greater than 220KDa was sliced and electroeluted[28] for 2 h at 25 mA, and extensively dialyzed against PBS.

Determination of LD_{50}

Briefly, 7 serial 10-fold dilutions of a sample were prepared, and 3 Balb/c mice (age, 4–6 weeks) were i.p. inoculated with 0.5 mL of each dilution. Results were observed within 3 days after inoculation, and the LD_{50} values were calculated according to the methods described by Reed and Muench[24].

Preparation of LcsT-specific rabbit polyclonal antiserum

Purified LcsT was inactivated by mixing 1 µL of 37% (w/v) formaldehyde with 0.3 mg of LcsT diluted in 0.3 mL of PBS and stirring the mixture (Thermomixer; Eppendorf, Germany) for 30 min at 37ºC[29]. The mixture was then extensively dialyzed against 10 mM Tris buffer, pH 7.5. The efficacy of inactivation was evaluated by LD_{50}[24] (as described above).

A rabbit was immunized by administering three 200 µg doses (first intra-dermal and other 2 intra-muscular) of inactivated LcsT in Freund's incomplete adjuvant on days 0, 20, and 50. After bleeding on day 60, controls of antiserum reactivity were performed by western blot[30]. Briefly, a sample of purified toxin was subjected to SDS-PAGE by using an 8% (w/v) under reducing conditions, and transferred into a nitrocellulose membrane, (pore size, 0.45 µm) (Amersham, USA), for1h to 12vol. Blotted membrane strips were then incubated with 0.05% (w/v) Tween 20 in PBS (PBS-T) containing 1% (w/v) bovine serum albumin (BSA) (Sigma, USA), for 1 h at room temperature (RT). The strips were then incubated with rabbit antiserum diluted 1/200 in PBS-T containing 0.1% (w/v) BSA, 1h at RT. The strips were washed 3 times with PBS-T, and incubated for 1 h at RT with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, USA), and appropriately diluted in PBS containing 0.1% (w/v) BSA. Following incubation, the strips were washed 3 times with PBS-T and treated with a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigam, USA).

The immunoglobulin (Ig) fraction was obtained from the antiserum by salting out with 37% (w/v) ammonium sulphate saturation[25].

Preparation and evaluation on cards of latex agglutination reagents

AJ10 latex particles (diameter, 0.1 µm; 10% (w/v) solid content) (Ikerlat, Spain) and K030 latex particles (diameter, 0.3 µm) (Merck, France) were coated with LcsT-specific rabbit Ig[0]. Coating trials were performed using 2 different mg protein:mg latex ratios for each type of latex assayed (0.050 and 0.060 mg Ig/mg AJ10 latex; 0.020 and 0.030 mg Ig/mg K030 latex). Coating was performed by incubating mixtures of latex particles with appropriate solutions of LcsT -specific Ig fraction in 0.1 M glycine and 150 mM NaCl, pH 8.2 over 2 h at 37°C to achieve mg protein/mg latex ratios. Subsequently, BSA (0.050 mg BSA/mg AJ10 latex and 0.025 mg BSA/mg K030 latex) was added, and the mixtures were incubated overnight at 4ºC and centrifuged at 20,000 rpm for 20 min at 4ºC. The pellets were resuspended in 100 mM glycine, 150 mM NaCl buffer (pH 8.2) to achieve a final particle concentration of 6 mg/mL. Particle size measurements were performed using a Zetasizer Nano particle analyser (Malvern Instruments, UK). Samples were subjected to ultrasound pulses (cycle duration, 0.5 min; amplitude, 50%; Hielscher UP 200S, Germany) to eliminate particle aggregates.

Agglutination tests were performed on cards by stirring mixtures of 30 µL of each of the latex reagents, whose preparation has been described in the previous paragraph, and 30 µL of sample dilutions in PBS. Agglutination was visually observed after stirring for 5 min. Serial 2-fold dilutions of each sample were prepared in PBS. Defined concentrations of purified LcsT diluted in PBS were assayed to estimate reagent sensitivity (detection limit). The detection limits of each reagent were established as the lowest toxin concentration at which agglutination was observed.

Correlation of culture progress with agglutination test results

The progress of increase in *C. sordellii* cell number in industrial cultures was evaluated in real time by optical density measurements at a wavelength of 600 nm (OD_{600}). Latex agglutination tests for the determination of TcsL concentration were performed with samples of filtered supernatants (0.45-µm mesh filter). For this purpose, the latex reagent that had shown the lower detection limit was used. Agglutination tests were performed on cards by stirring mixtures of 30 µL of the selected latex reagent and 30 µL of serial 2-fold dilutions of the supernatant in PBS, and agglutination was visually observed after stirring for 5 min.

RESULTS AND DISCUSSION

Purification of LcsT from *C. sordellii* culture supernatant

The first step in the TcsL purification from culture supernatant was performed by salting out with ammonium sulphate to obtain a TcsL-enriched fraction. Opti-

FULL PAPER

mum precipitation conditions were identified by performing precipitation tests on aliquots of C. sordelli culture supernatants by using different ammonium sulphate concentrations. The maximum toxin purification yield was achieved at 50% ammonium sulphate saturation (Figure 1).

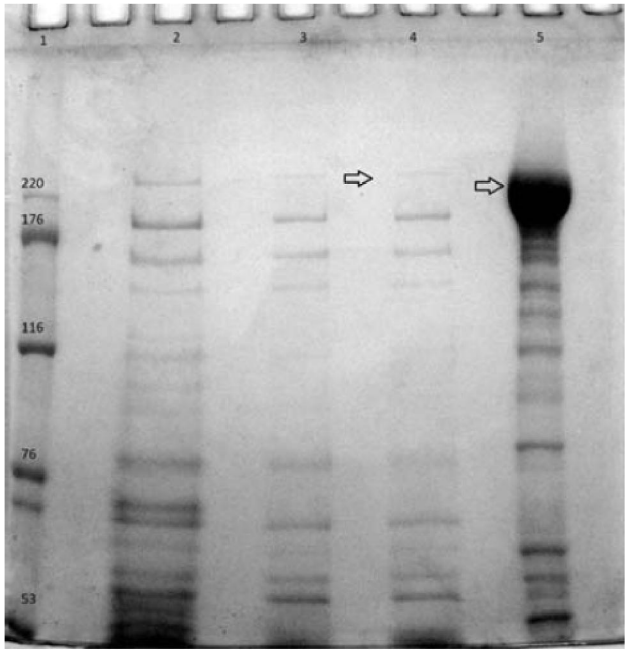

Figure 1 : JPEG- SDS-PAGE patterns of samples obtained after salting-out precipitation of culture supernatants with different concentrations of ammonium sulphate. Lane1: molecular weight markers (kDa); lanes 2–5, precipitates obtained at 0, 30, 40, and 50% of ammonium sulphate saturation concentrations, respectively. The line 5 is distorted by the amount of protein. Arrows noted bands are sent for analysis by MALDI-TOFF. SDS-PAGE 8% under reducing conditions stained with Coomassie Blue R-250.

Then the band greater than 220KDa was excised from the gel and analyzed by mass spectrometry (MALDI-TOF/TOF). Data from mass spectrometry (MS) and MS/MS identified the protein band as C. sordelli TcsL (data not show). Once the identity of the toxin was confirmed, and in order to achieve a preparation of higher purity, TcsL was electroeluted from preparative SDS-PAGE 8% of TcsL-enriched fraction. The purity of the electroeluted toxin was evaluated by SDS-PAGE 8% (Figure 2), here a single band was observed, showing that the toxin is pure.

Furthermore, as a quality control test, dissociation to acid pH of TcsL was performed, based on the studies of Daniel E. Voth et al.[37]. The purified TcsL was

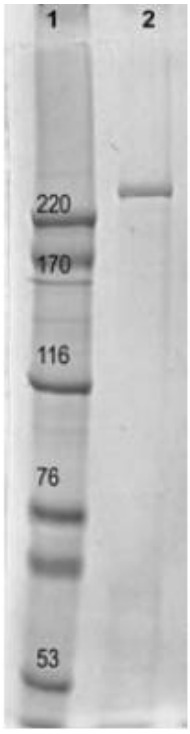

Figure 2 : JPEG-Characterization by SDS-PAGE of electroeluted TcsL. Lane 1: SDS-PAGE pattern of molecular weight marker (kDa), lane 2: electroeluted TcsL (30 µg). SDS-PAGE 8% under reducing conditions stained with Coomassie Blue R-250.

incubated for 30 min with 100 mM ammonium acetate buffer, pH 3.8, and then subjected to SDS-PAGE 10%. In Figure 3, it can be seen that there is a disassociation of TcsL in several proteins of lower molecular weights in agreement with the description of Voth el al.

A larger sample of purified TcsL was prepared using 2 L of *C. sordellii*. culture supernatant and was used for immunization and the remaining experiments.

Preparation of LcsT-specific rabbit polyclonal antiserum

Inactivation of the toxin used as immunogen was confirmed by LD_{50} experiments.

The specificity of rabbit anti-TcsL antiserum was assesed by western blot. For this purpose the antiserum was evaluated against purified TcsL (Figure 4) and against *C. Sordellii* culture supernatant concentrated by salting-out at 50% ammonium sulphate (Figure 5), blotted into nitrocellulose strips. In Figure 4, a band observed at> 220KDa, confirm the serum reactivity to purified TcsL. In Figure 5, a unique band of reactivity is observed at> 220KDa, this demonstrates that the rab-

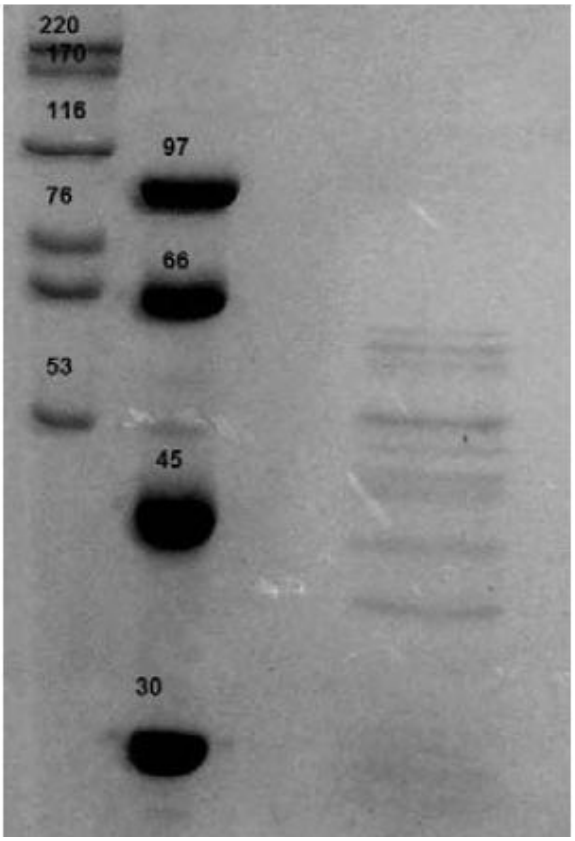

Figure 3 : JPEG-Characterization by SDS-PAGE of dissociation to acid pH of TcsL. Lane 1: SDS-PAGE pattern of molecular weight marker (kDa), Lane 2: SDS-PAGE pattern of molecular weight marker (kDa), lane 3: dissociated TcsL (30 µg). SDS-PAGE 10% under reducing conditions stained with Coomassie Blue R-250.

bit antiserum recognized specifically the TcsL among other proteins present in the complex mixture of culture supernatant.

Preparation and evaluation on cards of latex agglutination reagents

The detection limits of the latex agglutination reagents were determined by assaying the agglutination of 2-fold dilutions of 0.8 mg/mL purified TcsL. The lower detection limit was found for each particular reagent was 8 and 62 ng/mL for 0.060 mg Ig/mg AJ10 latex, and 0.020 mg Ig/mg K030 latex, respectively. The tests below were performed with the reactive. 060 mg Ig/mg AJ10 latex because it is the lower limit of detection provided.

Latex agglutination reagent was evaluated against three 0.45 µm-filtered culture supernatants. Arbitrary units of agglutination (+) were plotted against log (1/dilution) for each sample. Agglutination results for 0.060

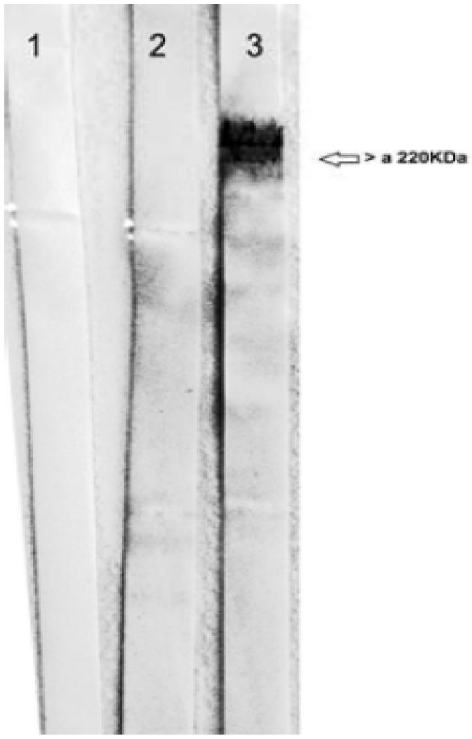

Figure 4 : JPEG-Western blot analysis of rabbit antiserum reactivity of purified TcsL. Lane 1: conjugate control; lane 2: pre-immune serum (dilution 1/100); lane 3: hyper-immune serum (dilution 1/200).

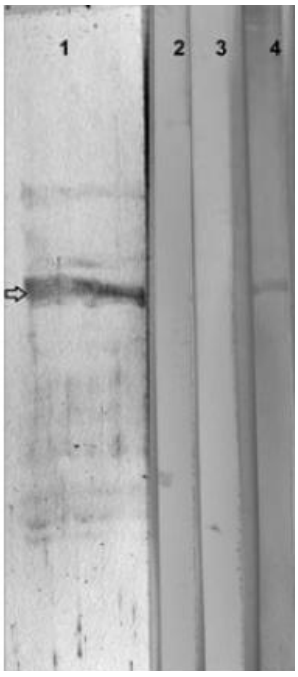

Figure 5 : JPEG-Western blot analysis of rabbit antiserum reactivity of culture supernatant *C. sordellii*. Lane 1: Blotted membrane strips stained with Amido Black; Lane 2: conjugate control; lane 3: pre-immune serum (dilution 1/100); lane 4: hyper-immune serum (dilution 1/200). The arrow indicates the TcsL.

FULL PAPER

mg Ig/mg AJ10 latex are presented in Figure 6. A prozone-like effect was observed for culture supernatant 3. Nevertheless this effect did not affect the performance of the test, since the samples were always assayed at least with 10 serial 2-fold dilutions. As positive control purified TcsL was used, and as negative control the reagent was tested against culture medium to verify absence of agglutination.

Specificity was analysed by testing cross-reactivity with cultures of C. novyi B, C. septicum, C. tetani, C. botulinum types C and D, C. perfringens C, and C. perfringens D. No agglutination was observed in any of these cases (data not shown).

Correlation of culture progress with agglutination test results

Experiments were performed in real time to evaluate the progress of cell growth in cultures by OD_{600} measurements as well as its correlation with the TcsL concentrations in supernatants by latex agglutination tests. The reagent prepared with AJ10 latex (0.060 mg Ig/mg latex) exhibited the lowest detection limit and was therefore selected for trials in industrial bioreactor conditions. Samples of culture supernatants were taken from an industrial bioreactor at different times, then OD_{600} measurements and latex agglutination tests were performed to monitor culture growth and toxin production. The results for 3 different batches are presented in Figure 7. As can be seen in Figure 7, the behaviour of the culture in terms of TcsL production is erratic, despite the attempted similar culture conditions; probably due to non-controlled parameters in the culture.

The absence of direct correlation between TcsL concentration and time or cell growth demonstrates that these parameters are not useful for determining the time at which culturing should be stopped. Furthermore, in 2 cases, it was observed that toxin concentrations detected by latex agglutination continued to increase even after the plateau for OD_{600} was reached. These findings emphasized the requirement of having a test capable of measure the status of the toxin concentration in each reactor on a real time basis. We demonstrated that the latex reagent developed was an efficient tool for real-time determination of the optimal culture harvesting time.

About quality control issues, several lots of reagent were prepared and assayed with successive 2- fold dilutions of purified active toxin. For each lot of reagent, the minimal concentration of purified toxin which caused agglutination was determined as the detection limit of the reagent. The purified toxin solution and the diluents constitute the quality controls that must be used for each set of assays to periodically verify the agglutination of the purified toxin and the absence of agglutination for the diluents in the industrial environment, along with the reagent batch control against matrix (culture medium). The results of this assay will not be extrapolable along with all the culture process. The reagent does not ag-

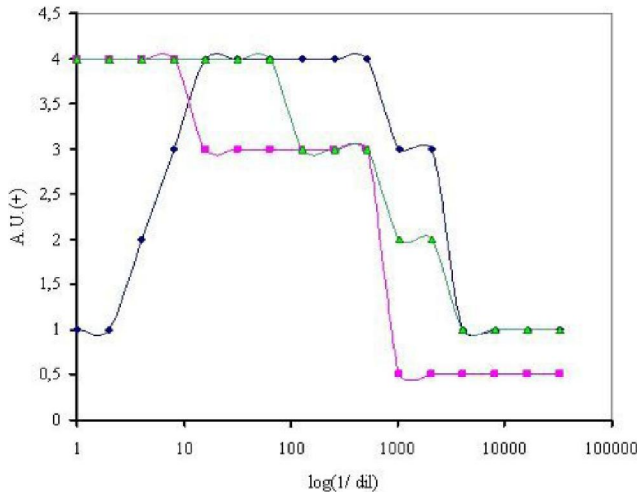

Figure 6 : JPEG- Evaluation on cards of latex agglutination reagents. Latex agglutination results, Arbitrary Units of agglutination [(A.U.)(+)] vs. dil^{-1} Reagent AJ10-0.06 with culture supernatants 1 (Δ), 2 (■) and 3(◊).

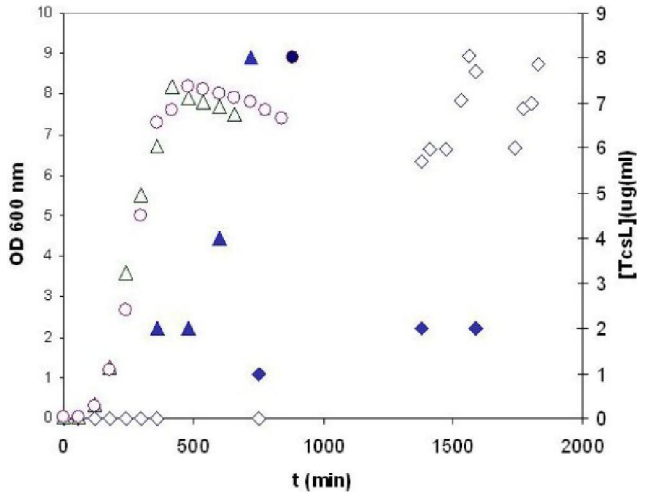

Figure 7 : JPEG- Correlation of culture progress with agglutination test results. Correlation of OD_{600} (left, open symbols) and [TcsL](µg ml^{-1}) (right, closed symbols) vs. time (min) for 3 different cultures.

glutinate neither with samples of the matrix (culture medium at t= 0) in the absence of the toxin or with samples of other clostridial toxins.

We suggest that, to establish the moment of harvesting of each industrial culture of *C. sordellii* containing the optimal TcsL production, the culture should be monitored with the reagent herein described, at intervals of 1-2 hours until the concentration of toxin is no longer increasing. Continuing the culture beyond this point is not recommended, because the toxin can be partially o totally proteolitically destroyed.

The industrial production of clostridial toxins is an important issue because these toxins are used to prepare veterinary vaccines. The control methods now used for optimization of toxin yield have several disadvantages, specifically, they are labour intensive, expensive, slow, and require the use of experimental animals. Few detection methods have been described for LcsT or other clostridial toxins, either for diagnostic or industrial uses[0-36]. However, the method described here is rapid; inexpensive; may be used next to the reactor; provides immediate results; does not require sophisticated equipment or skilled personnel; and is consistent with the trend to reduce, refine, and replace animal use. In this respect, the large volume of experimental animals required for the development of clostridial veterinary vaccines has increased the priority given to the development of *in vitro* potency- and safety-testing methods[38]. The latex reagent described here represents an important step towards achieving that objective as improving production yields is a necessary step towards improving the potency and safety of animal vaccines. This paper describes a semiquantitative method, which is not intended to replace the *in vivo* semiquantitative method now in use, but, instead, it serves as a complement thereto and offers as the most important advantage the possibility of decision making, based on semi- quantification in real time of the content of toxin in the culture.

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