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Optimization of Epsilon Toxin Production by Vaccine Strain of *Clostridium perfringens* Type D in Fermenter

A. K. Rai, V. K. Chaturvedi, T. G. Sumithra, S. C. Sunita, B. Joseph, M. S. Murugan, S. Pradhan

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Abstract Enterotoxaemia, an economically important overwhelming disease of small ruminants is prevented by epsilon toxoid vaccination. The cost for larger scale toxoid production is high that can be decreased by elevating the *in-vitro* expression of this toxin. In the present study an attempt was made to raise epsilon toxin production by use of fermenter and improvement of cultural media. The conditions such as amount of inoculums adaptation of *C. perfringens* type D to various quantities of media, speed and period of agitation, and maintenance of temperature and pH were standardized in fermenter. Simultaneously growth media components commercially available liver and beef extract powder were replaced with fresh caprine heart and liver tissue. Subsequently epsilon toxin titre of the trypsinised harvest was determined in mice after 18 h of incubation it was found that fermenter based cultivation could produce double titre of toxin compared to commercial media in stationary culture. Moreover the replacement of commercially available liver and beef extract powder with fresh caprine heart and liver tissue could produce two times greater toxin titre compared to ordinary production media. Thus the present study provides a potential cursor for achieving high yields of epsilon toxin titres thus for bulk production of toxoid based enterotoxaemia vaccines.

Keywords Enterotoxaemia, Fermenter, Epsilon toxin, *Clostridium perfringens*.

A. K. Rai*, Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, South Civil Lines, Jabalpur 482001, India
e-mail: ajay500rayss@gmail.com

V. K. Chaturvedi, Principal Scientist and Head, Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, Bareilly 243122, India
e-mail: vndchaturvedi@rediffmail.com

T. G. Sumithra, Scientist, Division of Marine Biotechnology, Central Marine Fisheries Research Institute, Ernakulam North P. O., Kochi 682018, India
e-mail: sumithravet@gmail.com

S. S. Chougule, Livestock Development Officer, Veterinary Dispensary, Sudhagadh, Pali, Raigadh 4102005, India
e-mail: sunitavet@gmail.com

B. Joseph, Assistant Professor, Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Navania, Vallabhnagar, Udaipur 313601, India
e-mail: dr.binzy@gmail.com

M. S. Murgan, Assistant Professor, Veterinary University Training and Diagnostic Center, Thiyagarajar Engineering College Road, Thirupparankundram, Madurai 625005, India
e-mail: drmsmadurai@gmail.com

S. Pradhan, Assistant Professor, Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, South Civil Lines, Jabalpur 482001, India
e-mail: shee1811@gmail.com
*Correspondence

Introduction

C. perfringens is responsible for one of the most

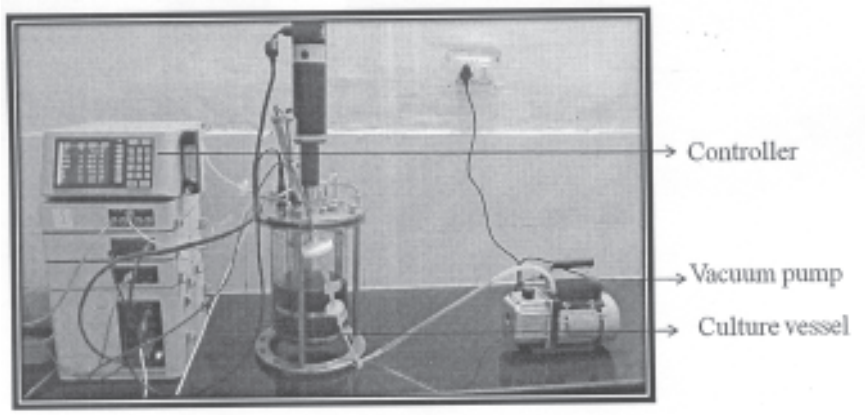

Fig. 1. Assembly of fermenter.

economically significant devastating infection among small ruminants called enterotoxaemia [1]. The rapid lethal progression makes the treatment impossible or impractical in most of the cases [2]. However, the disease can be effectively prevented by epsilon toxoid vaccination [3]. Due to a short term of immunity provided by these vaccines, a double initial vaccination is currently recommended for both sheep and goats, followed by a booster every year in sheep and every 3-4 months in goats [4]. In addition to this all the pregnant animals should be vaccinated during the fourth month of pregnancy [5]. The huge population of small ruminants and lengthy administration schedule of toxoid thus demands a very large scale production of toxoid vaccines. However, the vaccine production can be economized to a great extent by elevating the antigenic mass i.e. epsilon toxin. This underscores the need to embark upon a production system where high toxin titre with little inputs is possible.

When greater yields of bacterial or cell culture are needed, a single fermenter can be used to increase yield with the added advantage that it eliminates the requirement for added bench floor space and labor [6]. Though elaborate equations have been developed to describe agitation kinetics, mass transfer and energy transfer of fermenter, very little has been published regarding the use of fermenter in vaccine production [7]. Keeping these facts, in the present study we aimed to evaluate the use of fermenter to achieve high titers of epsilon toxin for economizing the bulk production of toxoid vaccines.

Materials and Methods

A highly toxigenic strain of *C. perfringens* type D procured from Division of Biological Standardization, IVRI, Izatnagar was used in the present study. Characterization of the strain was done by morphological, cultural and toxigenic properties. Molecular characterization was performed by PCR using *etx* specific primers (F–5'AAG GAT CCA AGT TTA GCA ATC GCA TCA GC3' ; R–5'TAC CTC GAG TTA TTT TAT TCC TGG TGC C3').

Preparation of production media for fermenter

Three liters of production media was prepared (liver extract 1%, beef extract 1%, peptone 2%, sodium chloride 0.1%, dipotassium hydrogen phosphate 0.4%, pH 8.2) in the fermenter vessel and autoclaved in vessel itself.

Assembly of fermenter

The fermenter (BIOFLO 110, New Brunswick Scientific, Edison, New Jersey, USA) was assembled by connecting controller with nitrogen cylinder (air in-

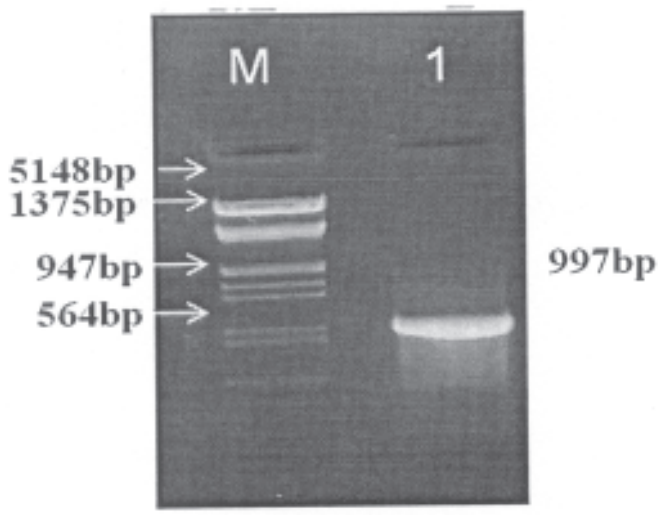

Fig. 2. PCR amplification of *C. perfringens* type D *etx* gene. Lane 1: Amplified *etx* gene, Lane M: Lambda DNA ladder.

let), pH pump and fermenter vessel (Fig. 1). Air inlet was then connected with 0.2 µm filter and outlet with 0.1 µm micron filter. pH pump was connected to NaOH and HCl bottles. Fermenter vessel was also connected with heating jacket, condenser and pH probe.

Operation of fermenter

To optimize the growth and epsilon toxin production, standardization of conditions such as amount of inoculums, adaptation of *C. perfringens* type D to various quantities of media, speed and period of agitation, and maintenance of temperature and pH were done. Finally the standardized protocol was as follows. First, for gradual adaptation of the organism *C. perfringens* type D was grown in 10 ml Robertson's cooked meat medium for 12 h which was then serially transferred to 100 ml, 500 ml and then again back to 100 ml with 12 h incubation in between each transfer. Fermenter vessel with sterilized media was adapted to 37°C which was then supplemented with 0.2% sterile glucose at the rate of 25 ml/L. Gas production was used as indicator of the growth. The initial agitation was carried out at 50 rpm for 30 min starting from 4 h of inoculation. Subsequently the medium was stirred at 100, 150 and 200 rpm each for a period of 30 min then kept constant at 200 rpm. Temperature and pH were kept constant at 37°C and 7.0 respectively and 0.2% glucose was added every 2 h interval. Excess gas produced during the growth was removed from the vessel by connecting to a vacuum pump at regular intervals. After 18 h, pH of the medium was adjusted to 8.0 and 0.25% trypsin was added and kept 37°C for 1 h without agitation.

Collection of harvests

After activation harvests were collected by siphoning in sterilized flask and purity was checked by wet film examination, blood agar slant and gram staining.

Cultivation of *C. perfringens* type D in stationary culture

The same production medium used in fermenter experiment was incubated at 37°C in an ordinary stationary incubator after inoculation of the seed strain. Production flasks were removed after 18 h of incubation and growth was tested for purity by microscopic and cultural examination. Simultaneously in attempt to increase the epsilon titre in static culture another media was prepared in which commercially available

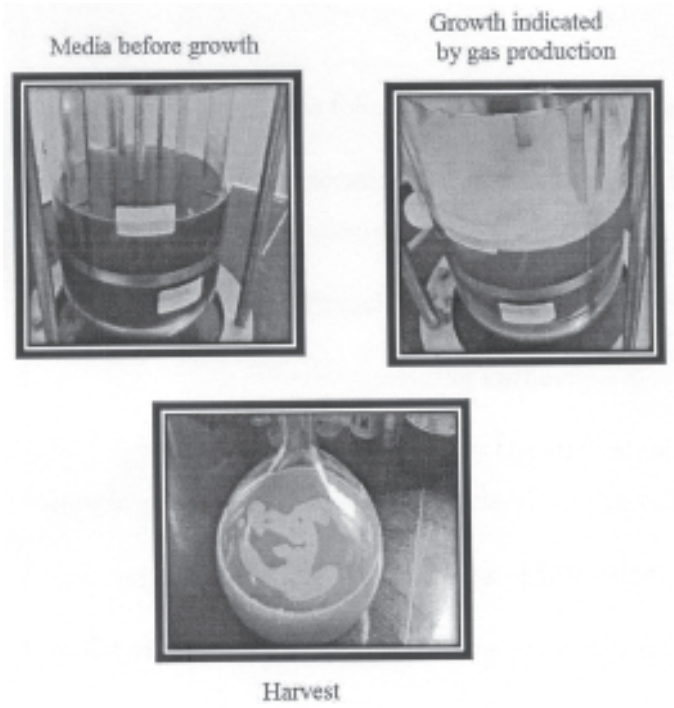

Fig. 3. Stages of growth in fermenter.

liver extract and beef extract powder was replaced by fresh caprine heart and liver.

Determination of epsilon toxin titre

After centrifugation of 50 ml harvest from all three types of cultures (fermenter culture and cultures grown in two different media), clear supernatant was collected. Minimum lethal dose per ml of the product was determined by injecting 0.1 ml of different dilutions of supernatant in sterile normal saline intravenously into two healthy adult mice. The highest dilution which kills both mice within 24 h is noted as the titre of the toxin.

Results and Discussion

During characterization, *C. perfringens* type D showed typical morphological and cultural properties. Molecular characterization yielded desired 997 bp product (Fig. 2). In purity test, growth in both stationary cultures and fermenter vessel was uncontaminated. In fermenter, an active growth was started within 2 h of incubation (Fig. 3). At the same time, it took about 3 h in stationary culture to produce tentatively same amount of gas. The titre of epsilon toxin of fermenter harvest was 4000 MLD/ml while that of stationary culture with commercial media was 2000 MLD/ml. At the same time, titre of epsilon toxin in production media prepared with fresh caprine heart and liver tissue was also 4000 MLD/ml.

In order to reduce the cost for larger scale production of enterotoxaemia epsilon toxoid vaccine a dense growth of the organism as well as the expression of epsilon toxins in ample amounts are required. Many useful microorganisms are grown on an industrial scale using fermenter. But, the ultimate test needed in passing fermenter for vaccine production is that whether it provides a reliable and efficient production of required antigen [7]. Since epsilon toxin is the most important antigen in enterotoxaemia vaccines estimation of this toxin titre was used for analyzing the difference between fermenter and stationary based cultivation of *C. perfringens*.

The growth standardization of different strains of veterinary bacterial vaccines has been done by various workers and may be an effective mean to economize the vaccine production with consistency of products. Standardization of *Pasteurella multocida* P52 growth in fermenter was done by Misra [8]. After standardization of various factors such as level of dissolved oxygen, phase of growth and amount of inoculums, Nenkov et al. [9] showed the simplicity of fermenter based cultivation of *Vibrio cholerae* in bulk production of cholera vaccines. Additional the worth mentioning observation was of Sun et al. [10] who showed that large quantities of antidiarrhoeal vaccine containing of divalent K88, K99 antigens of *Escherichia coli* could be provided by using a small fermenter. Similar work on the anaerobic organism in fermenter for the production of black quarter vaccine has been done by Cameron et al. [11]. Similarly Scorza et al. [12] developed a high yielding production process of outer membrane particles from *Shigella sonnei* using fermenter. In agreement with these findings, our experiment also showed that there is better growth of *C. perfringens* in fermenter (shown by gas production and epsilon toxin titre) after the standardization of various factors. Moreover, it was found that the titre of epsilon toxin produced in fermenter (4000 MLD/ml) was just double to that produced in the same media during stationary cultivation (2000 MLD/ml). However there is a need to further standardize on various physical factors and the composition of media. It should be noted that in our studies we did not attempt to optimize the media in fermenter vessel for maximum toxin production as we got only 4000 MLD/ml. But, the replacement of commercially available liver and beef extract powder with fresh caprine heart and liver tissue could produce two times greater toxin titre compared to earlier media showing that there is chance of better epsilon production by optimizing the media of inoculation. Nevertheless the double titre compared to static culture indicates the potentiality of fermenter for bulk production of toxoid based enterotoxaemia vaccines. So these results, or better, can be expected in future using fermenter technology on enterotoxaemia toxoid vaccine production.

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