Short Communication

Optimization of the In-Vitro Growth of Clostridium perfringens type D

Ajay Kumar Rai, Vinod Kumar Chaturvedi*, Thangalzhy Gopukumar Sumithra, Sunita Chougule, Bincy Joseph, Murugan Shanmugam

Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar - 243122, Uttar Pradesh, India
*Corresponding author: vlchaturvedi@mail.com

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In order to overcome the economic difficulties associated with large scale production of enterotoxaemia vaccine, an attempt was made to optimize the in-vitro growth of Clostridium perfringens type D, so as to elevate antigenic mass of the vaccine. Maximum growth density was obtained in the production medium supplemented with 0.2% sucrose and 0.2% vitamin mixture with pH adjustment (7.5-8.0) after 8h of incubation. Growth density in these conditions was about two times more, than the growth at ordinary conditions. Among sugars tested (glucose, sucrose and dextrin), sucrose was the better growth substrate. When pH of media was controlled to 7.5-8.0 after 8h of incubation, growth was approximately one and half times more compared to same medium with uncontrolled pH. Mixture of vitamin B complex and C showed a stimulatory effect when supplemented with sugars. The optimum temperature was found to be 41°C. Sodium chloride had the utmost effect at 0.75% concentration. Tween-80 and sodium acetate were proved as inhibitors.

Enterotoxaemia is a disease of great economical importance for sheep and goat farming worldwide (Niilo, 1980; Kriek et al., 1994), and has third rank among the various causes of death in small ruminants of India (Singh and Prasad, 2009). It can be well prevented by epsilon toxoid vaccine prepared from Clostridium perfringens type D culture (Jansen, 1967). Double initial vaccination is currently recommended for both sheep and goats, followed by a booster every year in sheep (Blood et al., 1983) and every 3-4 months in goats (Smith and Sherman, 1983).

India has the second largest goat population and third largest sheep population in the world (Annual Report, 2010-II), so requires a large scale production of enterotoxaemia vaccine. The vaccine production can be economized to a great extent by elevating the antigenic mass which requires dense growth of C. perfringens type D. Therefore in the present study, attempt had been made to optimize effect of various physical and chemical variables on the growth of C. perfringens type D so as to have dense growth of this organism.

A highly toxigenic strain of C. perfringens type D procured from Division of Biological Standardization, IVRI, Izatnagar was used in the present study. Sterilized Robertson’s cooked meat (RCM) media (Robertson, 1915) was used as the basal media to which various chemical agents were added to check the effect on growth. Chemicals such as glucose (20%), sucrose (20%), dextrin (20%), mixture of minerals viz. Iron, Magnesium, Zinc and Copper, mixture of vitamin B complex and C, tween-80, sodium chloride (10%) and sodium acetate were prepared and sterilized by filtration.

Chemicals such as 0.2% glucose, 0.2% sucrose, 0.2% dextrin, 0.5% mineral mixture, 0.2% vitamin mixture (final concentration in the medium is given in %), various concentrations of tween-80 (0.1%, 0.2%, 0.3%, 0.4% and 0.5%), sodium chloride (0.3%, 0.75%, 1%, 1.25%, 1.5% and 2%) and sodium acetate (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) were added to RCM. All were inoculated with 10% actively growing overnight culture of Clostridium perfringens type D and incubated at 37°C for 24h.

At the same time, with the aim of finding out the optimum physical conditions, the inoculated basal media are incubated under different temperature (37°C, 40°C, 41°C and 42°C) for various incubation periods (18h, 24h, 36h and 48h). To know whether there is any importance of pH maintenance of media during the growth, pH of media was adjusted 7.5 to 8.0 with IN NaOH at the end of 8 hrs of incubation. At the end of the incubation, O.D. of each culture was taken at 570nm.

Among the economically important bacterial diseases affecting small ruminants of India enterotoxaemia holds the first rank (Singh and Prasad, 2009) and accounts for 150 outbreaks, 1556 cases and 533 deaths only in 2009 (Annual Report, 2010-II). In order to reduce the cost for large scale enterotoxaemia vaccine production by elevating the antigenic mass in bacterial culture an attempt was made to optimize the in-vitro growth of C. perfringens under different physical and chemical conditions.

The maximum growth density was obtained in the production medium supplemented with 0.2% sucrose and 0.2% vitamin mixture with pH adjustment after 8h of incubation. The least growth was in those media supplemented with 0.2% dextrin without pH adjustment in between incubation. Further details of the results are given in Figures 1-5.

Among various sugars tested, sucrose was proved as better growth substrate which was similar to the report of Sacks (1983). But in contradictory to this, Fuchs and Bonde (1957) had reported that glucose was better than disaccharides as an energy source for C. perfringens. Further, vitamin also showed a...
stimulatory effect when added with these sugars. Interestingly, when pH of these media was controlled after 8h of incubation, growth was approximately one and half times more compared to same medium with uncontrolled pH indicating the need for pH maintenance of media during the cultivation of this pathogen. On increasing the incubation period for more than 18h, there was no significant difference in growth. However, incubation temperature had marked effect on growth. Though it was reported that the optimal temperature of C. perfringens is in the range of 43-47°C (Doyle, 2002) but the maximum growth was obtained at 41°C. On increasing the temperature up to 41°C there was a simultaneous increase in growth density, but after that further increase caused a decline in the growth. Morries et al (1996) reported that sodium chloride can enhance the availability of soluble proteins as a result increase the yield of B. thurigensis. However in the present study, sodium chloride was not giving a significant influence on growth of C. perfringens, though when used at 0.75% a slight increase could be observed. The same authors also used Tween-80 to increase the nutrient uptake of B. thurigensis. While it was observed that Tween-80 was having negative effect on C. perfringens. Still, this effect was found to be decreasing on increasing the concentration. In order to check the effect of organic salts, sodium acetate was taken as a model. It showed negative effect on growth but, a slight increase in growth could be observed when added at 0.2% compared to control. Similar to our observation Juneja and Thippareddi (2004) also demonstrated the inhibitory effects of organic salts on growth of C. perfringens.

Figure 1: Comparison of growth on media with pH control and without pH control

Figure 2: Effect of incubation period

Figure 3: Effect of incubation temperature

Figure 4: Effect of sodium chloride concentration
In conclusion, the present study suggests that in vitro growth of *Clostridium perfringens* can be maximized by growing them in a production medium supplemented with 0.2% sucrose and 0.2% vitamin mixture and adjusting pH of the medium to 7.5-8.0 after 8h of incubation. This will help to elevate the antigenic mass during the production of enterotoxaemia vaccine hence, cut down its production cost.

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