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A Comparative Study on The Production of Concentrates of Anthrax Live Spore Vaccine in Roux Flasks and in Fermenter

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Abstract: Forty batches of anthrax spore vaccine were produced by bioreactor and by Roux flask, 20 batches were obtained from each system. The number of doses produced in the flask system was found 10 times more than those obtained by the bioreactor. It is recommended to continue producing this vaccine by the Roux flask while research will go on to improve suitable conditions in the fermenter in order to obtain high doses and to avoid the drawbacks of the flask system.

Key words: Anthrax, vaccine, rouxflasks, fermenter

INTRODUCTION

Anthrax is a highly lethal infectious disease of mammals including human[1-3]. It is fatal when *Bacillus anthracis* spores enter the body through abrasion in the skin, by inhalation or by ingestion[4].

Anthrax is zoonotic to which most mammals, especially grazing herbivores are considered susceptible. Human infections result from contaminated animals or animal products, there are no known cases of human-to-human transmission.

The live spore vaccine that has been developed by Sterne[5] is most widely used to prevent animals from the disease. A rough variant of the virulent *Bacillus anthracis* was derived from culture on serum agar in an elevated CO_2 atmosphere. The variant named 34F2, it was incapable of forming capsule and it was subsequently found to have lost the PX02 plasmid which codes for capsule formation[6].

The licensed human vaccine Anthrax Vaccine Absorbed (AVA) prepared from culture supernatants of toxogenic, unencapsulated strain of *Bacillus anthracis*, protects animals against inhalation anthrax[6]. Numerous animal studies have shown that the primary immunogen in AVA is the Protective Antigen (PA), a component of anthrax toxins[8-10].The mechanisms of protection induced by AVA remain unknown but recent studies in rabbits have shown that protection is correlated with antibody to PA[10], antibody to PA confers passive protection on rabbits and guinea pigs[11]. The aim of this work was to carry out a comparative study of anthrax live spore vaccine production by the Roux flask and the fermenter system in order to adopt the system that yield high number of doses for the routine work.

MATERIALS AND METHODS

Organism: *Bacillus anthracis* Sterne strain 34 F2

Media: Lablmcoo medium (Oxoid) was mixed with the following ingredients (g L^{-1}): Lablmcoo medium15.0, yeast extract 2.5, K2HPO4 0.131, CaCL2 0.033, MgSO4 0.020, MnSO4 0.011 and Agar (Merck) 0.5

15 g L^{-1} of caesin enzymatic hydrolysate (Oxoid) was prepared with the same ingredients that have been added to Lablmcooo. Nutrient agar was also prepared with the same ingredients

Fermenter: IBT Bioreactor was used for production of live spore's anthrax vaccine. The following parameters were adjusted throughout the whole experiment for the vaccine production:

Working volume	2 L
Temperature	37°C
pH	7.2
Aeration for growth	300 cm/14 sec
Aeration for sporulation	maximum
Inoculums size	5 %

The fermenter was set up and sterilized at 121°C at 15 Pound for 45 min. All accessories of the fermenter were sterilized together.

Preparation of the working seed for the fermenter: The working volume seed was prepared by inoculating colonies (grown on DST agar) of the master seed into 100 mL nutrient broth (Oxoid) then incubated at 37°C overnight. Sterility of the seed was checked by direct

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microscopic examination and by culturing onto blood agar plate which incubated at 37°C for 48 h. The pure culture with non-granulating cells was selected as inoculums into the fermenter.

Fermenter follow-up: The medium was kept under observation for 2 days before inoculation of the fermenter by the working seed to check sterility; sterility was verified by absence of medium turbidity and the stability of the already adjusted pH. The seed which constitute 5 % of the working medium was inoculated into the fermenter, other parameters were adjusted as prementioned. After good growth of the organism in the fermenter vessel, aeration was increased from 300 cm/14 sec to maximum to have good sporulation. Samples for sterility were examined daily by direct microscopy and culture onto blood agar plates. When 90 % or more of vegetative cells undergo sporulation the culture was harvested in batch form system. 5g L^{-1} of phenol was added to the harvests as preservative and to kill the non-sporulating vegetative cells. Batches were preserved at room temperature for 2 weeks then the count of the live spores was determined by viable tube count (most probable method) in a semi-solid (0.1 %) nutrient agar.

Preparation of anthrax vaccine in Roux flasks

Preparation and testing of the working seed: Vial of the stock was reconstituted and inoculated in several slants (approximately 10 mL) of sporulation agar then incubated at 37°C for 72 h and stored at 4°C. The slant was tested for purity by culturing on nutrient agar plates and in nutrient broth (Oxoid). Wet films from samples were also checked for absence of motility.

Method of the vaccine production: Seed lots were cultured on solid media formulated to promote sporulation of the organism. The solid medium formula was: 50 g tryptic digested casein (Merck),10 g yeast extract (Oxoid), 0.1 g $CaCl_2.6H_2O$(Merck), 0.01 g $FeSO_4.7H_2O$ (Merck), 0.05 g $MgSO_4.7.H_2O$ (Merck), 0.03 g $MnSO_4.4H_2O$ (Merck), 5.0 g K_2HPO_4,1.0 g(Merck), KH_2PO4 (Merck), 22 g agar (Oxoid), 1000 mL of distilled water. The ingredients were dissolved in water with appropriate amount of heating; the pH was adjusted to 7.40, sterilized by autoclaving, dispensing into Roux bottles and cooled in horizontal position. After the agar was solidified, excess liquid was removed aseptically and the bottle was incubated at 37°C for 2 days to dry and check sterility. Volume of 2 mL of the working seed was spread across the agar in Roux bottles then incubated at 37°C for 72 h to let the organism to grow on the surface of the nutrient agar and to undergo sporulation. When more than 90 % sporulation (apparent by microscopic examination of aseptically extracted loopful) the batches were harvested. The amount was harvested using 10 mL sterile distilled water per bottle and then check for purity.

RESULTS

Twenty batches of anthrax vaccine were produced by fermenter using Lablmcoo (Oxoid) and casein enzymatic hydrolysate (Oxoid). As shown in Table 1, high viable spore count (1.0X 10^{10} spores m L^{-1}) was obtained in batch 16 and the low count (5.0X 10^7 spores m L^{-1}) was gained in batch 4, Lablmcoo was used in the two batches.The average viable count produced by the fermenter was 3X $10^{8.6}$ spores m L^{-1}).

When the vaccine was produced on the solid medium in Roux flasks (Table 2), the viable count in the 20 batches was 1.0 X 10^9- 1.0X ^{11} spores m L^{-1} with average of 3.0X $10^{9.7}$ spores m L^{-1}.

DISCUSSION

Anthrax vaccine production was based on the seed-lot system[5]. In the fermenter it was produced in batch system unlike the other bacterial vaccines (Hemmorhagic septicaemia, Black quarter vaccine) which were produced by continuous system. Different media were used as a nutrient source of the bacteria but the most common are Lablmcoo and casein enzymatic hydrolysate. From our results (Table 1), the viable count of different batches was variable despite of the constant parameter adjusted in the fermenter, they were found variable even within the same media. No clear reason(s) for these inconsistent results, but the quality of the working volume seed might be a factor in determining the density of spores in one batch. However, some batches (not included in this study) revealed a very high spores density at harvest time when examined by the microscope, but two weeks later all the spores were almost vanished, they were neither be seen by the microscope nor be detected in the tube after the viable count. This phenomena was not noticed when the flask system was used but in a very occasional frequencies some flasks showed no bacterial growth on the medium surface or no spore development took place despite of the similar condition of incubation. It was either a human error by missing inoculating the flask with the bacterial seed or unexplainable reason that inhibits sporulation. Spores contain a large amount of divalent cations predominantly Ca^{2+} but with significant amount of Mg^{2+}, Mn^{2+} and other

Table 1: Viable count of live spores of anthrax vaccine produced by the fermenter

Batch No	Medium used	Viable count
1	Lablmcoo	$1.0X\ 10^{8}$
2	Lablmcoo	$1.0X10^{8}$
3	Lablmcoo	$5.0X10^{8}$
4	Lablmcoo	$5.0X10^{7}$
5	Caesin Enzymatic Hydrolysate	$5.0X10^{8}$
6	Caesin Enzymatic Hydrolysate	$1.0X10^{9}$
7	Caesin Enzymatic Hydrolysate	$1.0X10^{9}$
8	Caesin Enzymatic Hydrolysate	$7.0X10^{9}$
9	Caesin Enzymatic Hydrolysate	$1.0X10^{9}$
10	Lablmcoo	$5.0X10^{9}$
11	Lablmcoo	$1.0X10^{10}$
12	Lablmcoo	$7.5x10^{8}$
13	Lablmcoo	$5.0X10^{8}$
14	Lablmcoo	$2.5X10^{9}$
15	Lablmcoo	$1.0X10^{9}$
16	Lablmcoo	$1.0X10^{10}$
17	Lablmcoo	$2.5X10^{9}$
18	Lablmcoo	$5.0X10^{8}$
19	Lablmcoo	$2.0X10^{9}$
20	Lablmcoo	$1.0X10^{9}$

The mean viable count = $3.X10^{8.6}$ spore m L^{-1}

Table 2: Viable count of different batches of anthrax vaccine produced by Roux flasks

Batch No.	Viable spores count
1	$7.0X10^{9}$
2	$1.0X10^{9}$
3	$5.0X10^{9}$
4	$5.0X10^{9}$
5	$1.0X10^{9}$
6	$4.0X10^{9}$
7	$1.0X10^{10}$
8	$1.0X10^{10}$
9	$1.0X10^{10}$
10	$1.0X10^{10}$
11	$1.0X10^{10}$
12	$1.0X10^{10}$
13	$3.0X1.0^{10}$
14	$5.0X10^{10}$
15	$5.0X10^{10}$
16	$1.0X10^{10}$
17	$1.4X10^{10}$
18	$5.0X10^{10}$
19	$1.0X10^{11}$
20	$3.0X\ 10^{10}$

Mean viable count = $3.0X\ 10^{9.7}$ spore m L^{-1}

ions as well[12]. The minerals incorporated in the nutrient medium were selected for spore's development; all these minerals were in good quality that supposed to enhance sporulation beside other factors such as increase aeration rate. The process of sporulation might not be completed and those spores developed seemed to be immature. However, the initiation of sporulation in response to nutrient deprivation requires the product of several genes, called Spo O genes. The exact environmental stimuli which cause sporulation are unknown and may acquire a combination of several factors[13], we think that solid media enhance transformation of vegetative cell to spore more frequent than liquid media, this might be to rapid nutrient depletion in the former.

The viable count in batches produced by Roux Flasks (Table 2) was almost consistent and the mean viable count of these batches was almost 10 times the average count of batches produced by the fermenter. Though bioreactor is a convenient system for mass vaccine production, especially those maintained by continuous system and it requires no more hands but the result of this study indicated that the number of doses of anthrax spore vaccine is higher when Roux flasks was used and this give the advantage of Roux flask system to the fermenter.

For the time being it is recommended to produce the live spore anthrax vaccine by the Roux flask system and simultaneous research will go on for production of this vaccine by the fermenter system to improve conditions that promote gaining high spore's density, more number of doses and to avoid the drawbacks of flask system.

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