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Identification of host-derived *Hansenula polymorpha* DNA in the recombinant hepatitis B vaccine by using a dot-blot hybridization method

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In the recombinant hepatitis B (HB) vaccine produced by using yeast as an host organism, the residue of the host-derived DNA in more than permissible amount can cause undesirable effect on vaccine recipients. In this study, the host-derived *Hansenula polymorpha* Deoxyribonucleic Acid (DNA) in the recombinant hepatitis B vaccine produced by CJ Pharmaceutical Plant, Republic of Korea was identified by using a dot blot hybridization method. First, a single strand hybridization probe was prepared by a template extracted from *Hansenula polymorpha* DNA and was labeled with d-UTP-DIG by using PCR technique followed by testing of the labeling efficacy of the probe. The host-derived DNA from recombinant HB vaccine samples was extracted by using phenol/chloroform method to obtain DNA pellets whereas standard DNA with known concentration was prepared in different dilutions. Then hybridization of vaccine samples and standards was done on hybridization nylon membrane by using a prepared hybridization probe labeled with dUTP-DIG in the UV cross linker, hybridization oven, sealable plastic bag and several kinds of washing, detecting and developing solutions/buffers. Finally, DNA contents in vaccine samples were quantified on hybridization membrane by comparing the intensity of specific color signals with those of the standard DNA in different concentrations. No apparent color signal was observed in all 3 test samples of recombinant HB vaccine thus indicating the total absence of host-derived DNA in recombinant HB vaccine and total safety of it for human use.

INTRODUCTION

Advances in molecular genetics and nucleic acid chemistry now enable genes coding for natural biological active proteins to be identified, analyzed in detail, transformed from one organism to another and to express under controlled conditions so as to synthesize efficiently the specific protein for which they coded [1]. For production of recombinant hepatitis B (HB) vaccine, hepatitis B surface antigen (HBsAg) expressed gene which is characterized by specific nucleotide sequence in each strand of the double stranded DNA molecule with 678 base pairs that code for HBsAg protein was isolated and propagated by insertion into a suitable vector, pH-HBs, with an aid of highly specific restriction endonucleases and ligase enzymes. This vector was then introduced into the chromosomal DNA of *Hansenula polymorpha* yeast cell and individual clones that carry the desired gene was selected and propagated in mass culture, followed by steps of purification process to obtain the desired protein. During process of recombinant HB vaccine production in the CJ Pharmaceutical Plant, Republic of Korea, first the chromosomal DNA of *Hansenula polymorpha* yeast cell is fragmented by high pressurein cell disruption procedure using a homogenizer. Most of the host-derived DNA were removed by serial steps of purification process i.e pH precipitation, ultracentrifugation and gel filtration processes [2]. Since the residue of the host-derived DNA in more than permissible amount in final vaccine product can cause undesirable effect on vaccine recipients, the amount of host

derived-DNA in recombinant HB vaccine needs to be determined and to be within specific limits [3]. In this study, an appropriate method for identification of host-derived DNA in the recombinant HB vaccine produced at the CJ Pharmaceutical Plant was established with an aim to assure the quality and safety of the final product .

MATERIALS AND METHODS

This is a laboratory-based study carried out at the Viral Vaccines Laboratory, CJ Corporation, Ichon City, Republic of Korea. First, a single strand hybridization probe was prepared by a template, labeled with d-UTP-DIG by using PCR technique followed by testing of the labeling efficacy of the probe. The host-derived DNA in recombinant HB vaccine was extracted by using phenol / chloroform method to obtain DNA pellets. Commercially available standard DNA with known concentration was prepared in different dilutions.

Hybridization of 3 vaccine samples and DNA standards was done on hybridization nylon membrane by using a prepared hybridization probe labeled with dUTP-DIG using a UV cross linker, hybridization oven, sealable plastic bag, prepared washing, detecting and developing solutions/buffers. Finally, DNA contents in vaccine samples were quantified on hybridization membrane by comparing the intensity of specific color signals with those of the standard DNA in different concentrations. Detailed procedures were as follows;

Preparation of a single strand hybridization probe labeled with dUTP-DIG

First, a mixture containing a template extracted from *Hansenula polymorpha* DNA hexamer (TTAGGG) dNTP-DIG and Klenow fragment (DNA polymerase I) was under PCR program at 37^{o}C for 18 hours. The PCR mixture was treated with 4 M lithium chloride and 100% ethanol to precipitate the desired DNA probe which was kept in Tris-EDTA (TE) buffer at -20^{o}C. The labeling efficacy of the above probe was confirmed by comparing with a commercially available standard DNA probe of 5µg/µl. Serial dilutions of sample and standard probes in 10^{-1} to 10^{-7} (i.e. equivalent to 500 to 0.005 pg/µl) was carried out, followed by applying onto the hybridizationnylon membrane and keeping in the UV crosslinker for 30 seconds (fig.1). Then the above nylon membrane was incubated in blocking solution at room temperature for 30 minutes, in antibody solution (i.e antidigoxygenin-Ap) for 60 minutes and in washing buffer for 15 minutes. After discarding washing buffer, the membrane was equilibrated in the detection buffer for 2 minutes and incubated in color developing mixture (NBT/ BCIP solution) for 30 minutes. Finally the reaction was stopped by TE buffer. The specific (yellow) color signals were observed on the hybridized nylon membrane.

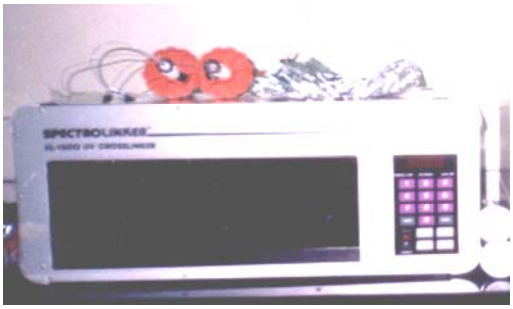


Fig.1. UV cross linker for fixation of DNA to the hybridization nylon membrane.

Extraction of host-derived DNA from the recombinant HB vaccine samples by using phenol/ chloroform method

Seven hundred microliters of vaccine sample was added into an equal volume of phenol, followed by vigorous vortexing, centrifugation at 13,000 rpm for 15 minutes and transferring supernatant into a new microfuge tube. Equal volume of phenol was added into the supernatant followed by vigorous vortexing, centrifugation and transferring supernatant into a new micro-fuge tube. One-tenth volume of 3 % sodium acetate was added into the supernatant followed by vigorous vortexing. After

adding of 2 volumes of 100 % ethanol into the sample, it was kept on ice for 30 minutes, followed by centrifugation at 13,000 rpm for 15 minutes and collecting the DNA pellets. Finally, one volume of 70% ethanol was added, followed by vortexing and kept at 60°C for 30 minute to obtain a dried DNA pellet. Before application onto the hybridization nylon membrane, DNA pellets were resuspended in TE buffer to make a final DNA concentration of 10 µg/µl followed by incubation at 37°C for 30 minutes and kept on ice.

Preparation of standard DNA into different dilutions

Commercially available standard DNA 5 ng/ µl of Invitrogen, Life Technologies, was diluted to 50, 100, 200, 400, 800 and 1600 pg / 2 µl by using 2xSCC buffer.

Application of samples and standard into the hybridization nylon membrane

Two microliters each of 3 vaccine samples and DNA solutions in 6 different dilutions (i.e 1600, 800, 400, 200, 100, 50 pg per 2 ml) were applied onto the nylon membrane which was then kept in a UV cross linker for one minute, followed by introducing into the heat sealable plastic bag containing 2 ml of hybridization solution and 20 µl of probe labeled with d UTP-DIG. After sealing the bag, it was incubated in the hybridization oven at 42°C for 1 hour (Fig. 2).

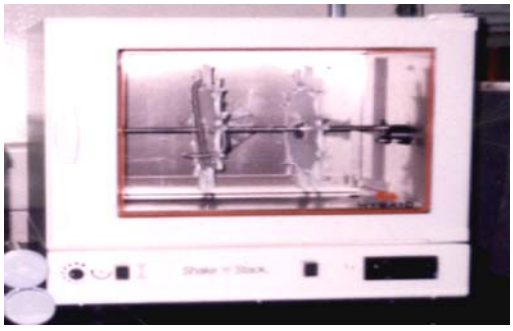


Fig.2. Hybridization oven used for hybridization of standards and vaccine samples with aprepared probe labeled with dUTP-DIG

Then the membrane was removed and transferred into the new bag containing 2 ml of hybridization solution, followed by tight sealing and kept in the hybridization oven at 42°C for 48 hours.

Detection of specific signals for DNA contents

The hybridization membrane was then removed and put into a 50 ml falcon tube containing 20 ml of 2xSCC and 0.1% SDS mixture and kept in the hybridization oven with rotatory shaking at 37°C for 15 minutes. The serial treatment of the mixture with 0.5xSCC+0.1% SDS mixture, blocking solution containing maleic acid buffer, antibody solution of anti-digoxyginin-Ap, Dig-washing buffer and detection buffer i.e NBT/BCIT solution from Roche [4], was followed by developing the mixture at 37°C for 30 minutes (or) till the appearance of specific colored signals. Finally, the reaction was stopped by using TE buffer to obtain a hybridization membrane with the colored signals representing the DNA content in the DNA standards and vaccine samples.

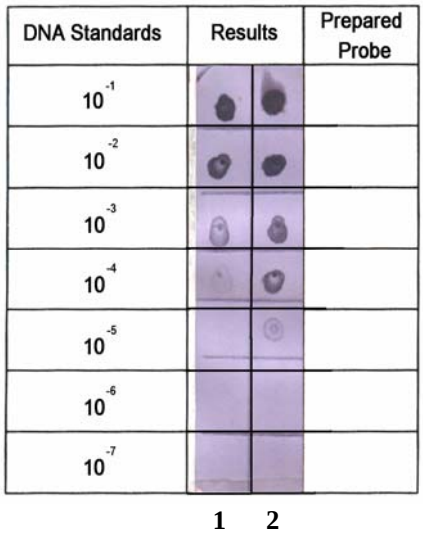
RESULTS

Figure 3 illustrates specific colored signals on the hybridization membrane in the probe labeling efficacy test, representing the different dilutions of DNA standards and a prepared probe. Specific colored signals were identified up to 10^{-4} (i.e.500 pg/ml) in the standards and 10^{-5} (i.e.50 pg/ml) in the sample probe, indicating the acceptable efficiency of the prepared probe labeled with dUTP-DIG.

Hybridization nylon membrane with specific colored signals of DNA was illustrated in Figure 4. The standard lane showed respective colored signals at different dilutions of DNA contents whereas no apparent colored signal was observed at sample lane with 3 different test vaccine samples, thus indicating the absence of DNA in the vaccine samples of recombinant HB vaccine.

DISCUSSION

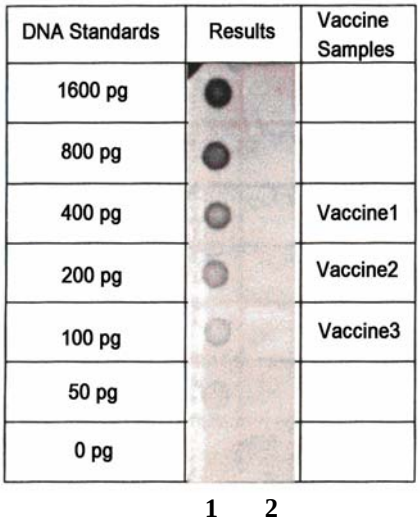
Contamination of recombinant HB vaccine with biologically active extraneous DNA from host-derived transformed *Hansenula polymorpha* yeast cell is undesirable and is particular concern for the possible presence of potentially oncogenic DNA. The WHO Study Group on Biologicals mentioned that the possibility of risk associated with heterogeneous contaminating DNA in a product derived from recombinant yeast cell line is negligible when the amount of such DNA is 100 pg or less in a single dose given parenterally [3]. Therefore, the amount of plasmid or host-derived DNA in each lot of vaccine should be determined and be within specified limits. The analysis method should be sensitive and reliable. In most of the GMP standard vaccine-producing countries, the maximum acceptable levels of DNA per vaccine dose and an appropriate method for determination is approved by the National Control Authority (NCA) [3].



Lane 1 = DNA standards , Lane 2 = prepared probe

Fig. 3. Specific colored signals on the hybridization nylon membranes in the probe labeling efficacy test

In this study, before detecting the host-derived DNA in vaccine sample by hybridization procedure, a probe labeling efficiency test for the prepared probe labeled with dUPT-DIG by using PCR method was carried out. Since the colored signals elicited by the prepared probe was comparable to those of the standards and passed the efficacy test, the result of further hybridization procedure was assumed to be accurate and valid (Fig. 3).



Lane 1=6 DNA standards, Lane 2=3 vaccine samples

Fig. 4. Hybridization nylon membrane with specific colored signals of DNA in 6 different dilutions of DNA standards and 3 samples of recombinant hepatitis B vaccine

After hybridization procedure, different dilutions of DNA standards showed specific colored signals in linear decreasing order on hybridization nylon membrane. However, vaccine samples elicited no signals which indicated the absence of host-derived DNA in the vaccine (Fig. 4). Therefore, the amount of host-derived DNA present in the recombinant hepatitis B vaccine produced from *Hansenula polymorpha* yeast cell was in accordance with specifications recommended by WHO.

It could be concluded that there was no possibility of risk associated with the host-derived DNA in recombinant HB vaccine produced by the CJ Pharmaceutical Plant from which the technology of recombinant HB vaccine production was transferred to the Department of Medical Research (Lower Myanmar) (DMR,LM) in 2003 under the EDCF loan of US$ 12.6 millions from

Republic of Korea. The recombinant HB vaccine was successfully developed in GMP standard HB Vaccine Plant in Myanmar by scientists from DMR(LM) under supervision of Korean scientists in 2004. This vaccine was also tested and recommended to be totally safe and immunogenic for prevention of hepatitis B viral infection [5, 6].

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