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DETERMINATION OF HEPATITIS B SURFACE ANTIGEN PARTICLES BY ELISA

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ABSTRACT

Hepatitis B surface Antigen (HBsAg) expressed in *Pichia pastoris* assemble into particulate structures. Enzyme-linked immunosorbent assay (ELISA) was used to determine these particles. The covalent attachement of enzymes to antibody molecules created an immunological tool possessing both high specificity and high sensitivity. The specificity, rapidity and inexpensive nature of ELISAs have insured their prominence in clinical and veterinary medicine.

KEYWORDS: Hepatits B, *Pichia pastoris*, ELISA, clinical and veterinrary medicine, Antigen

1. INTRODUCTION

Hepatitis B is caused by a highly contagious virus that infects the liver. It affects all age groups and can lead to liver disease, liver cancer and death in many of those afflicted. The virus is found in the blood and body fluids of infected people and can be spread through sexual contact, the sharing of needles or razors, from mother to infant during birth and by living in a household with a chronically infected person. The hepatitis B virus (HBV) is hardy and can live outside the body for several days [1].

Potentiometric determination of hepatitis B surface antigen in biological fluids was described [2]. Hepatitis B surface Antigen (HBsAg) was determined quantitatively by radio immunoassay and by Laurell electrophoresis in sera of 90 patients with acute hepatis B, 57 patients with chronic hepatitis B, and 154 HBsAg positive blood donors by Frosner *et al.* [3]. The sensitivities of three techniques used to detect serum hepatitis B surface antigen (HBsAg) were compared in 411 patients with various types of chronic liver disease by Chiaramonete *et al.* [4]. They found that counter immunoelectrophoresis proved an unreliable test. Two haemagglutination techniques were slightly less sensitive than radioimmuno assay but were more rapidly performed. Less sensitive techniques such as detection of hepatitis B surface antigen (HBsAg) and determination of HBsAg and anti-HBs were particularly unreliable in active liver disease where HBsAg titres were low [4].

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The covalent attachment of enzymes to antibody molecules created an immunological tool possessing both highly specificity and high sensitivity. The technique called ELISA (for enzyme linked immunosorbent assay) makes use of antibodies to which enzymes have been covalently bound such that the enzymes catalytic properties and the antibody's specificity are unaltered. Typical enzymes include peroxidases, alkaline phophatases and B-glycosidase, all of which catalyze reactions whose products are coloured and can be measured in very low amounts. Besides the ELISA test for Acquired Immuno-deficiency syndrome (AIDS), literally hundreds of clinically useful ELISAs have been developed. The rapidity and low cost running for ELISA tests at great appeal since time and money are usually at a premium, but it is the extreme sensitivity of ELISAs that really makes them important immunodiagnostic tools. New ELISA tests are marketed each year and many of them are rapidly replacing other methods.

The purpose of this research is to prepare soluble extract from *Pichia pastoris* cells and use ELISA to determine the concentration of Hepatitis B surface Antigen (HBsAg) particles in the extract.

2. MATERIALS AND METHODS

2.1 Preparation of soluble Extracts for ELISA

Aliquots of *Pichia* cells from International Centre for Genetic Engineering and Biotechnology (ICGEB) used corresponding to 100 OD_{600} were transferred into microfuge tubes with 5µl zymolase, mixed gently and incubated at 30 ^{0}C for 1 hour. The cells were pelleted by low-speed centrifugation (4000 rpm, 8 mins.) and washed with 0.5 ml 1 M sorbitol. Washed cells were resuspended in 1.5 ml lysis buffer containing 0.6 g zirconia glass beads (0.45 µm) and lysed by 10 one-minute bursts of vortexing at maximum speed, with chilling on ice for 1-min between bursts of vortexing at maximum speed, with chilling on ice for 1-min between bursts. The lysate was spun down and the supernatant collected. The concentration of Hepatitis B surface Antigen (HBsAg) particles in this lysate/sample (soluble extract) was determined by ELISA [5].

2.2 ELISA FOR SOLUBLE HBsAg

Two basic ELISA methodologies have been developed, one for detecting antigen (direct ELISA) and the other for antibodies (indirect ELISA). For detecting antigens such as virus particles from a blood or facal sample, the direct ELISA method is used. In this procedure the antigen is "trapped" between two layers of antibodies. The specimen is added to the wells of a microtiter plate previously coated with antibodies specific for the antigen to be detected. If the antigen (virus particles) is present in the sample it will be trapped by the antigen binding sites on the antibodies. After washing unbound material away, a second antibody containing a conjugated enzyme is added. The second antibody is also enzyme specific for the antigen so it will bind to any specific for the antigen so it will bind to any remaining exposed determinants. Following a wash, the enzyme activity of the bound material in each microtiter well is determined by adding the substrate of the enzyme. The color formed is proportional to the amount of antigen originally present.

Direct ELISAs have been developed for detecting hormones, drugs, viruses, and a variety of other substances or disease agents in human blood and other tissues. Hepatitis B surface antigen in soluble cell extracts were analyzed using Hepanostika ELISA kit from International Center for Genetic Engineering and Biotechnology, India. A strip containing 8 wells from the Hepanostika kit was removed and left on the bench top for 15 minutes to reach room temperature. The soluble extracts were diluted 1:1000 using 1 X PBST as a diluent. Aliquots of 100 were pipetid into the wells, the strip was taped on top and incubated for 37 ^{0}C for 1 hour sample aliquots ranging from 25µl-100µl were assayed according to the manufacturer's instructions; the TMB substrate was prepared by mixing equal volumes of the two component substrate from the kit in a tube. The tube

was covered with a foil and left at room temperature for 5 minutes. After one hour incubation was over, the contents of the wells was poured into the sink carefully. The wells were rinsed as follows. 1 XPBST was added to the wells and left for a minute. Wells were flipped over a wad of blotting sheets to empty them. The PBST rinse was repeated 4 times totally. After the final PBST rinse, 100µl TMB substrate was added into each well, the strip tapped and incubated for 30 minutes at 37 ^{0}C. Color changes developed in 25 minutes. The reaction was stopped with 10µl in H_2SO_4 and readings were taken using microplate reader at 450 mm (single wavelength).

3. RESULTS AND DISCUSSION

The readings from the microplate reader when aliquots of 25 ul, 50 ul, 75 ul, 100 ul were pipetted into wells A, B, C, D, E, F, G and H are shown in Table 1 and the readings from the microplate reader for standards are shown in Table2. Following measurement of the amount of antibody bound to a series of microtiter wells containing increasing concentration of antigen to be tested, was added, the amount of antibody bound can be determined and compared with the standard. Therefore, following the establishment of a standard curve, the HbsAg concentration in a sample can be estimated (Figure 1). Hepanostika ELISA Kit could be used to estimate the concentration of Hepatitis B surface antigens in a sample. This test is rapid and the cost is low. Hermann *et al.* [6] reported that supernatant from recombinant *P. pastoris* expression cultures could be used in ELISA assay and because of the normally high concentration of ScFv (single chain) fragments; even highly diluted supernatants provided a strong signal in this assay. Vassileva *et al.* [7] and Cregg *et al.* [8] showed that Hepatitis B surface antigen in *P. pastoris* assemble in particulate structures. Cregg *et al.* [8] and Himan *et al.* [9] reported that the Hepatitis B surface antigen expressed in P. pastoris as fusion protein retains its ability to form particles. In the light of all these, it was important to know if the ELISA Kit works for a novel source of antigen, which could be considered as a potential material for immunizing people at risk from Hepatitis B.

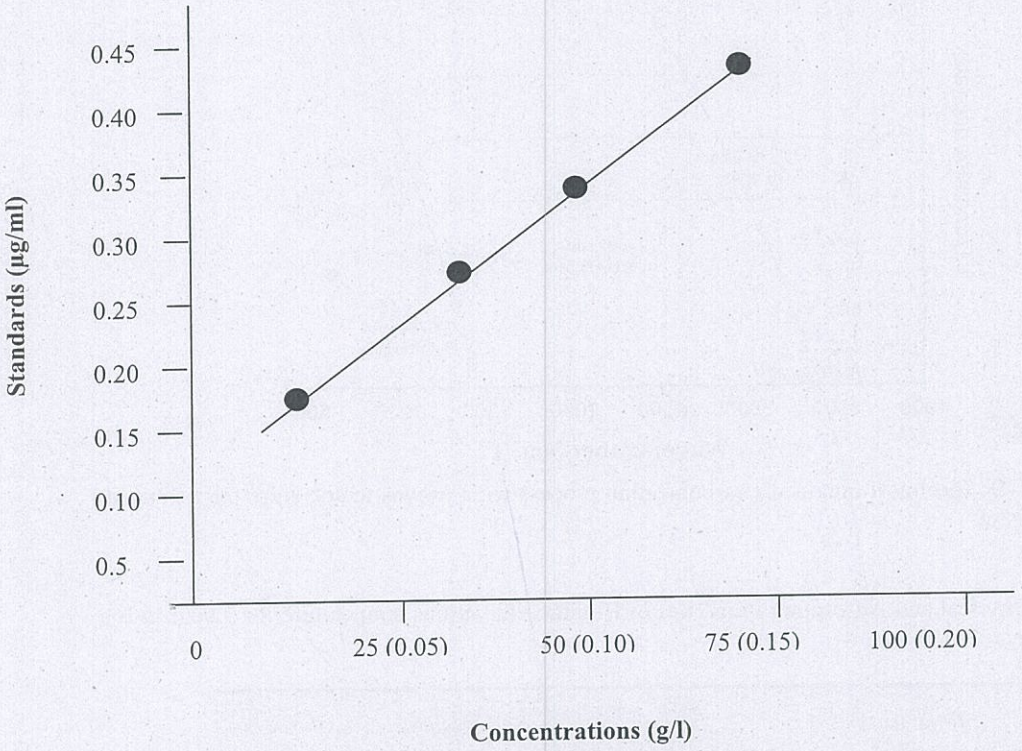
Table 1 Readings from microplate reader

	1	2	3	4	$\overline{x} \pm$ S.D
A	0.195	0. 074	0.290	0. 216	0.191 ± 0.0776
B	0. 381	0. 542	3.394	0. 079	1.099 ± 1.3354
C	0. 075	1. 491	3. 656	0. 077	1. 325 ± 1.4646
D	0. 311	0. 970	0. 120	0. 161	0. 391 ± 0.3420
E	0. 448	0. 106	0. 066	0. 199	0. 205 ± 0.1485
F	0. 918	0. 994	0. 854	0. 468	0. 809 ± 0.2027
G	0. 079	2. 708	1. 227	2. 370	1. 596 ± 1.0339
H	0. 690	1. 823	0. 055	0. 083	0. 663 ± 0.7163

Remark: Control (Blank) = 0.093

Table 2 Readings from microplate reader for standards

	100 mg/ml of HBsAg standards	Amounts
A	0. 182	25µl
B	0. 287	50µl
C	0. 332	75 µl
D	0. 390	100µl



4. CONCLUSIONS

In conclusion, this work demonstrated that the Hepatitis B surface Antigen particles were determined by ELISA. This test is rapid and the cost is low compared to other tests. It can be used in clinical and veterinary medicine.

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