Current Trends in Biotechnology and Pharmacy
Vol. 9 (3) 266-272 July 2015, ISSN 0973-8916 (Print), 2230-7303 (Online)

Comparison of the Efficacies of Different ELISA formats in Sero-detection of Antibodies to HPV 16 E7 Oncoprotein

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Abstract

Different formats- the indirect ELISAs with either peptide or the E7 whole protein as antigens and the more recent GST-E7 capture ELISA have been employed in sero-epidemiological studies for the determination of HPV infection. Further, several studies have attempted to correlate sero-prevalence with prognosis and/or early diagnosis of cancer. This study compares the efficacy of ELISA in sero-detection when different forms of the E7 antigen *viz.,* E7 peptides, E7 whole-antigen, and GST-E7 fusion protein are used. The sera samples collected from hospital-visiting women were grouped into two categories- "suspect" negative (n=20; from women 18 years of age and negative for HPV 16 L1 antibodies determined by the VLP ELISA) and "suspect" positive (n=40; from women whose corresponding Pap smear samples showed presence of HPV 16 DNA and serum showed sero-positivity for HPV 16 L1 antibodies). The suspect negative sera showed absorbance readings below the respective cut-off points in all formats of the ELISA. However the suspect positive samples showed varied results with ~4%, ~20% and ~73% of samples showing absorbance readings above the cut-off in the E7 Peptide, E7 whole-antigen and GST-E7 capture ELISAs respectively. The results indicate that the GST-E7 based capture ELISA might be best suited for use in the sero - detection of antibodies in individuals with unknown disease or infection status.

Key words: HPV16 E7, E7 Peptide ELISA, E7 whole-antigen ELISA, GST-E7 Capture ELISA

Introduction

The Human Papillomavirus genotype 16 (HPV 16) is the predominant high-risk HPV type associated with squamous cell carcinoma (Cervical cancer, Head and neck cancer etc.). The hallmark of an HPV associated cancer is the long-latency from infection to full-blown cancer. Therefore, early prognosis is quintessential to the effective management of the disease. Mandatory screening through Pap smears, prompt follow-up and treatment is proven to effectively check disease progression and mortality in developed nations (1). But in lesser-developed regions the skill and resources needed to emulate the management strategy are scarce. Hence there has been intense interest in adopting tests such as ELISA for prognosis and early diagnosis (2).

The HPV E6 and E7 onco-proteins have been suggested as potential serological markers of HPV associated cancer since tumorigenic cells show constitutive expression of these viral proteins (3). ELISAs reported for the sero-detection of E7 specific antibodies employ the E7 antigen as peptides (4), recombinant whole-antigen (5) or as fusion antigens, most notably tagged with Glutathione-S-Transferase (GST-E7) (6).

This article presents the evaluation of the ELISA for detection of E7 antibodies with respect to the use of different forms of the E7 antigen in the assay.

Materials and Methods

Serum samples: Serum samples analyzed in this study were collected from consenting women who visited the Sir Sundarlal Hospital, Banaras Hindu University. Sera for comparison were classified into "suspect positive" and "suspect negative" based on the criteria detailed below.

Suspect positive sera (n=45): a. Sera (n= 45) from donors who also tested positive for HPV 16 DNA in the DNA hybridization assay using the commercial Linear Array HPV Genotyping Test Kit from Roche Molecular system, USA in the corresponding cervical smear samples as described elsewhere (7).

b. The above sera that also showed positivity for HPV 16 major capsid protein L1 in the VLP based ELISA as described elsewhere (8)

Suspected Negative sera (n=20): Sera obtained from girls below the age of 18 years and showing no reactivity in the VLP-based ELISAs

Genes, vectors and bacterial hosts: Codon-optimized HPV16 E7 gene for expression in E. coli was obtained from GeneArt, Germany as a plasmid borne synthetic construct. Plasmid propagation and sub-cloning were performed in the host strain, E. coli Top 10 cells (Life Technologies™, USA). The E7 gene was sub-cloned in the commercial expression vector pGEX4T1 (GE Healthcare, USA) between *Eco* RI and *Not* I sites. Expression of the GST-E7 fusion protein was performed in the in *E. coli* BL21 cells (GE Healthcare, USA).

Expression and Purification of GST-E7**:** Sub-cloning was performed following standard procedures (9). Briefly, The E7 gene from Geneart™ was digested with the *Eco* RI and *Not* I restriction enzymes (New England Biolabs Inc., USA) and inserted into a pGEX4T1. The insert: vector ligations were performed using the Rapid Ligation Kit™ from Roche, USA. pGEX4T1/E7 clones were propagated in E. coli Top 10 cells after transformation of the chemically competent bacterial cells. Further, pGEX4T1/E7 plasmids were obtained from overnight bacterial cultures of transforming E. coli Top 10 cells using the Qiagen MiniPrep Kits™ as per the manufacturer's instructions (Qiagen, USA).

Recombinant E7 expression was carried out in the E. coli BL21 strain cells (GE Healthcare, USA) by induction with 1mM IPTG (Genie, Bangalore) at an OD600 of 0.6. After an induction period of 4 h at 37°C the cells were lysed using a sonicator (Vibra-Cell™ VCX-700, Sonics & Materials Inc., USA) in Potassium phosphate buffer (pH 7.2) containing 0.2M NaCl, 5mM DTT and 2mM EDTA. The GST-E7 from the cell-lysates was then purified by affinity chromatography using the Glutathione Sepaharose 4B matrix (GE Healthcare, USA) according to manufacturer's instructions. Determination of expression and analysis of purification was performed by protein electrophoresis (PAGE) following standard methodologies (9).

E7 protein and E7 peptides**:** HPV16 E7 was obtained by the proteolytic digestion of the affinity-purified preparation of GST-E7 protein using the thrombin cleavage kit from Novagen® Darmstadt, Germany. After digestion, the mixture was subjected to Glutathione Sepharose 4B affinity chromatography for removal of GST. The residual thrombin (biotinylated) was removed by affinity chromatography using Streptavidin-Agarose matrix.

Synthetic overlapping peptides (20mer with 13mer overlaps) of the full-length E7 protein were obtained from JPT Peptide Technologies, GmbH, Germany.

Indirect peptide and E7 ELISA: Maxisorp™ 96-well plates (Nunc; Thermo Scientific, USA) were coated overnight at 4°C with either HPV 16 E7 (for E7 ELISA) or with the Synthetic E7 peptides (Peptide ELISA) at the concentration of 200 ng / well/100µl in 0.05M carbonate buffer (pH 9.6). Blocking was done by incubation (1.0h) with 2% casein in phosphate buffer saline contain 0.05% Tween 20 (PBST) Sera for analysis (suspected positive and suspected negative) were added in triplicates at a dilution of 1:25. To facilitate the antigen-antibody binding, plates were incubated for 1h at 37°C. Bound antibodies were then probed with 1:5000 dilution of goat anti-human IgG peroxidase™ (ABD Serotec, UK). Between the ELISA steps plates were washed with PBST using a Microtitre plate Washer (BioRad, USA). The assay was developed with 3, 3, 5, 5-Tetramethylbenzidine Liquid Substrate system (Sigma-Aldrich, USA). Post development for 10 min, the reaction was stopped by the addition of 1.25N H_2SO_4. The absorbance at 450nm was measured in the Microtitre Plate absorbance reader (Beckman Coulter DTX 880 Multimode detector USA). Appropriate experimental controls, *viz.,* wells treated with influenza peptides (for peptide ELISA) and GST protein (for indirect E7 whole antigen ELISA) was incorporated in the assays. Commercial monoclonal antibody to HPV16 E7 (Santa Cruz, USA) served as the positive antibody control in the assays.

Glutathione capture ELISA: Maxisorp™ 96-well Microtitre plate wells were coated with 200ng/ well/of casein- glutathione in 0.05M carbonate buffer, pH9.6 and were incubated overnight at 4°C. Wells were blocked with 2% casein in PBST for 1.0h. Binding antigens, either GST-E7 or only GST at 200ng/well were added and incubated at 37°C for 1h. Sera for evaluation were added in triplicates at 1:25 dilution. The rest of the ELISA steps *viz.,* developing, stopping and recording absorbance was done as detailed above for the E7 protein and peptide ELISAs.

Determination of Cut-off value: As reported elsewhere (10), the cut-off value was decided as the average plus three times standard deviation of the absorbance values obtained for experimental negative sample in triplicates in the ELISAs. For the peptide ELISA wells coated in triplicates with long-peptides of influenza formed the experimental negative samples. For the E7 whole antigen indirect ELISA and GST-E7 capture ELISA wells coated in triplicates with recombinant GST alone represented the experimental negative samples.

Comparison of the ELISA results: The absorbance values in the ELISA were presented in a Box-and-Whisker plot using the Origin pro software.

Results

Purified proteins, GST-E7 and E7: The recombinant GST-E7 expressed and purified from the *E. coli* BL21 showed a 37.0 kDa product in SDS-PAGE. Recombinant E7, obtained as a thrombin cleavage product from the fusion GST-E7, subscribed to the size of ~12kDa (Figure 1).

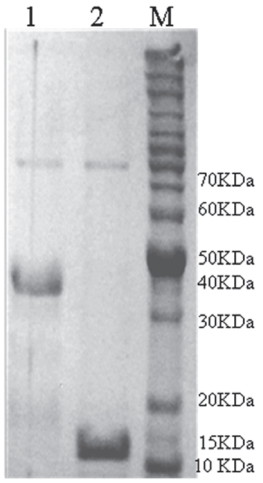

Fig. 1. SDS-PAGE profile Lane 1- Affinity purified HPV16 GST E7 protein; Lane 2: HPV16 E7 protein after thrombin cleavage; Lane M: Protein molecular-

Cut-off points: The cut-off point for the E7 peptide was A_{450} of 0.146 while the cut-off point for E7 whole-antigen and GST-E7 capture ELISAs was A_{450}= 0.148.

Comparison of the three ELISA formats: Out of the 45 suspect positive sera 2 (~4%), 9 (20%), and 33 (73%) samples showed positivity in the peptide, whole-antigen (E7) and capture (GST-E7) ELISAs respectively. None of the suspect-negative sera (20 samples) showed absorbance values higher than the cut-off in any of the ELISAs viz., Peptide, whole-antigen E7 or the capture ELISA.

Discussion

Approach of the comparative study: Different ELISA protocols have been reported (4, 5 and 6) for the detection of antibodies to HPV. Early studies involving the detection of antibodies to E7 have mostly been performed with indirect ELISAs using synthetic E7 peptides (13, 14). The ease of availability of synthetic peptides rather than the considerable effort involved in the generation of recombinant protein may have been the reason for the choice of antigen. E7 peptide ELISAs, even among cancer patients have usually reported seroprevalence of only about 30% of the evaluated cases (14).

Capture ELISA using recombinant GST-E7 generated from E. coli has been a more recent advent for serological detection of antibodies to E7 and other HPV proteins (6). Recent reports pitch for the GST- E7 capture ELISA as an economical yet specific assay for detection of antibodies against HPV proteins, including E7 owing to the presence of the GST tag (6). A recent article reports nearly 66% of the cervical cancer patients showing seroprevalence of antibodies to HPV proteins E6 and/or E7 (13). One of the reasons that may be accorded to the increased sensitivity of the GST fusion protein based capture ELISA is the antigen in holistic form *vis-a-vis* peptides.

Here, we have attempted to compare the effectiveness of the peptide ELISA, whole-antigen ELISA and GST-E7 based capture ELISA in sero-detection of antibodies. Available literature points at a distinct, positive correlation between seroprevalence of antibodies to HPV proteins and progression towards cervical cancer (15, 16). Further, it has been reported that persons with persistent infection often show antibodies to HPV proteins L1, E6 and E7. On this premise, the serum samples were grouped into suspect positive. And therefore these serum samples were from individuals whose cervical cytological samples showed the presence of HPV 16 DNA while also the sera contained antibodies to HPV 16 L1, the outer capsid protein.

Sexual activity has been known to facilitate HPV infection and transmission (11). Therefore the suspect negative samples constituted sera from young unmarried women (=also below 18 years) which also did not show L1 antibodies. With this grouping of serum samples used in the comparative study, we reasoned a call on the effectiveness of the each of the different formats of the assay could be made with better certainty.

The results of the ELISA as represented in the Box-and-Whisker plot (Figure 2) indicate that the capture ELISA is the best format for the detection of the sero prevalence. Nearly 73% of the suspect positive samples showed absorbance values higher than the cut off point for the ELISA. While the E7 whole antigen ELISA fared better than the peptide ELISA (20% vs 4%) the results are more in line with the previously reported studies (17). The high percentage of positivity for antibodies to onco-proteins E6 and E7 with individuals most likely harbouring HPV has been reported recently using the GST capture ELISA (15).

The large difference between peptide ELISA and the GST-E7capture ELISA in detecting antibodies may be attributed to the profound difference in the physical conformation of the antigens (peptide vs GST-E7). Linear peptides can be expected to discount largely or even completely the conformation specific antibodies. GST-E7 on the other hand can expectedly display E7 protein in a tertiary

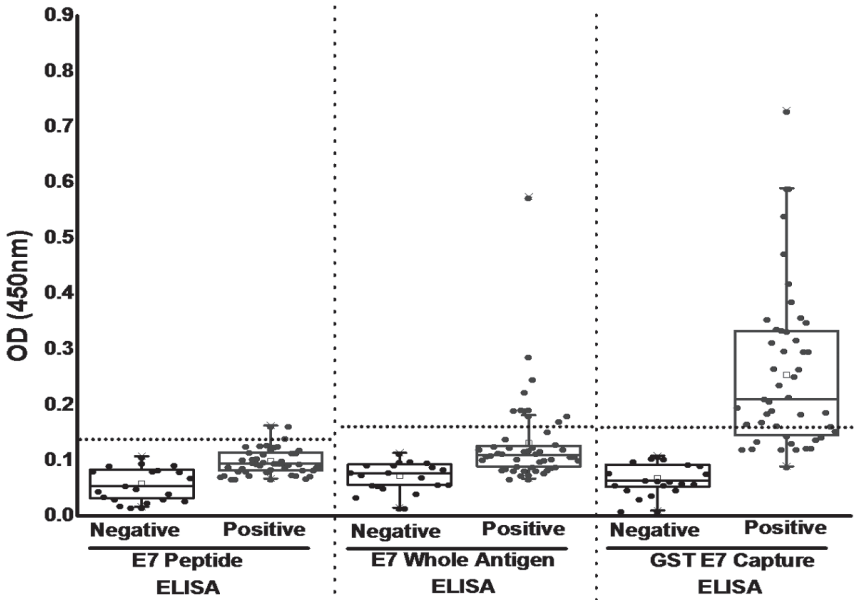

Fig. 2. Comparison of three ELISA formats for the determination of anti-HPV16 E7 antibodies in sera represented in a Box-and-Whisker plot. The graph depicts the spread of samples ordered based on the absorbance values. Whiskers and boxes represent interquartile regions, with the lower boundary of each box representing the 25th percentile and the upper boundary, the 75th percentile. The line inside the boxes represents the median value. The whiskers represent the 5th and 95th percentile. Points beyond the whiskers are samples that are outliers.

configuration and ensures fully accessibility for antibody binding in the capture ELISA. The low number positive samples in the E7 whole antigen ELISA relative to the capture ELISA need further investigation. However, it may be mentioned that the reported propensity of E7 to aggregate at near neutral pH (18) may have some bearing on antigenic form influencing the detection. Besides, it is also not uncommon for the distortion of antigen on binding to microtitre plate wells (19), especially with high-binding surfaces (MaxiSorp™).

Conclusion:

The results indicate that the GST-E7 based capture ELISA might be best suited for use in sero-detection of antibodies in individuals with unknown disease or infection status. Additionally the GST tag confers significant advantage in protein purification. Hence the GST-E7 based capture format of ELISA might find use in sero-epidemiological studies and probably a role in prognosis and/or early- diagnosis of cancer.

Acknowledgements

The authors are grateful to the management of the National Diary Development Board (NDDB), Anand, for providing the facilities to carry out this work. Author, Ravi Ranjan Verma, expresses his gratitude to the Indian Immunologicals Limited, Hyderabad for providing the opportunity and support for this study as a partial fulfillment for his PhD thesis.

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