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Seroprevalence of Antibodies to HPV L1 in a Limited Population study determined by the GST-capture ELISA

Ravi Ranjan Verma [a], Rajan Sriraman [b], NM Ponnanna [c], Samir Kumar Rana [c], Shikha Srivastava [d], JK Roy [d], Sadhana Gupta [e], M Madhanmohan [a] and V A Srinivasan[c*].

[a] Department of Biotechnology, Jawaharlal Nehru Technological University Hyderabad and R&D Centre, Indian Immunologicals Ltd., Gachibowli P.O., Hyderabad 500032, India.
[b] B13/166 Sonarpura, Varanasi 221001, India
[c] National Dairy Development Board, R&D Laboratory, Gachibowli P.O., Hyderabad 500032, India.
[d] Department of Zoology, Banaras Hindu University, Varanasi 221005, India.
[e] Department of Obstetrics & Gynaecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India.
*For Correspondence - srinivasanva1948@gmail.com

Abstract

Human papillomavirus (HPV) infection elicits an antibody response in most individuals, mainly to L1- the major capsid protein of the virus. The Glutathione S-Transferase (GST) capture ELISA was evaluated in a limited population study (n=187 women donors) for its suitability in determining the seroprevalence of antibodies specific to L1 of five high-risk HPV genotypes (16, 18, 31, 45 and 52). Serum samples were obtained from some of the hospital-visiting women aged between 18-60 years with an unknown history and/or status of infection. Most samples (42%) showed sero-positivity to HPV 45 (80/187) followed by sero-positivity to HPV 16 (32%), 18 (16.5%), 52 (10%) and a single (0.5%) positive sample for HPV 31. There was significant cross-reactivity in the samples across the assayed genotypes. The results suggest that the GST-capture ELISA might serve as an efficient alternative to VLP-ELISA in sero-epidemiological studies especially in the large-scale, preliminary, screening of individuals hitherto untested for HPV infection.

Keywords: HPV genotype 16, 18, 31, 45 and 52; GST-L1; GST-capture ELISA; Sero-positivity; VLP-ELISA

Introduction

Cancer of the uterine cervix or cervical cancer is the second most common cancer in women worldwide. Every year, 450,000 new cases are diagnosed and 220,000 patients succumb to the disease (1). It is now firmly established that infection with HPV of the high-risk genotypes, predominantly by the type 16 and 18 (2, 3) precludes cancer. HPV genotypes 16, 18, 31, 45 and 52 together are associated with more than 75% of cancers (4). The infection with the virus is common in majority of women who are sexually active. Although, the first encounter with the virus may occur at an early age coinciding with sexual maturity, the manifestation of the symptomatic disease is observed much later, usually after 35 years of age. In most individuals the virus is cleared spontaneously within months or a couple of years following infection. However, a small number of infected individuals fail to clear the virus and carry persistent infection; a significant proportion of these individuals acquire the disease (5, 6).

Considering the long latency from infection to cancer, early detection is the key to the management of the disease (7). Tests based on detection of the HPV DNA in the cervical smear samples provide a definitive answer to the

current status of the infection (presence or absence of the virus) but offer no clue to the history of infection (previous infections) (8). Besides, the current DNA detection tests are based on PCR and/or hybridization methods, which require considerable skill, training, instrumentation and complexities in both sampling procedures and testing.

ELISA technique, it may be argued, is relatively simple, better understood and has been in wide usage in clinical diagnoses. In view of the persisting serum antibodies to the major capsid protein L1 of HPV, in majority of infected subjects (9), serological diagnosis by ELISA is a promising tool for early detection of present as well as past infections. The major capsid proteins, L1, expressed in heterologous hosts, self-assemble into virus-like particles (VLPs) (10). Since VLPs are structurally near-identical to the native virus current vaccines for prophylaxis of HPV infection are based on VLPs produced from recombinant L1 proteins. Logically hence, the evaluation of vaccine efficacy is based on ELISA with VLPs as coating antigens (11, 12). The VLP based ELISA is also the current gold standard for HPV L1 serological studies (13). Recently, an alternate format namely the rapid capture ELISA based on the glutathione-S-transferase L1 fusion protein (GST-L1) expressed in *E. coli* was reported for the serological evaluation of HPV infection (13). The ELISA involved coating of microtitre well plates with glutathione coupled casein for capturing the GST-HPV16 L1 antigens present in the crude lysate of recombinant *E. coli*. The results of the capture assay, in their study, showed good concordance with the VLP ELISA in the determination of HPV 16 L1 specific antibodies in women. Since the production of GST-L1 antigens involve little downstream processing relative to the production of VLPs it offers a convenient alternative in sero-epidemiological and vaccine efficacy studies (14).

This paper reports the development of a standard ELISA protocol using the purified HPV16 GST-L1, rather than the earlier reported (13) use of crude lysates of bacteria expressing the protein, for the detection of L1 specific antibodies of the five high-risk HPV genotypes (16, 18, 31, 45 and 52).

Materials and methods:

Cloning, expression and purification of HPV major capsid protein : HPV16, 18, 31, 45 and 52 L1 genes, borne on a plasmid flanking with *EcoR*I and *Not*I restriction sites were chemically synthesized (GeneArt, Germany). The genes were sub-cloned into pGEX-4T-1 vector (GE Healthcare, USA) using the Rapid DNA Ligation Kit (Roche, USA) and subsequently transformed into competent *E. coli* BL21 cells. Recombinant *E. coli* BL21 cells were cultured in LB broth and were induced with 1mM IPTG at 0.6 OD_{600} for 4h. Post induction the cells were harvested and later re-suspended in 50mM potassium phosphate buffer containing 200mM NaCl, 1mM EDTA, 1mM DTT, 2mM ATP and 5mM $MgCl_2$. The cell mass was then lysed by sonication (Sonics Vibra cell, USA) and the respective recombinant L1 proteins of the HPV genotypes (16, 18, 31, 45, 52) were purified by affinity chromatography using the Glutathione Sepharose® 4B affinity media as per the manufacturer's manual (GE Healthcare, USA). Analysis of purification was performed with 10% SDS-PAGE and by Western blotting with anti-HPV16 L1 monoclonal antibody (Camvir™; Novus Biologicals, USA) and anti-GST polyclonal antibody (GE Healthcare, USA).The concentrations of the proteins were determined using the Bicinchoninic acid (BCA) protein assay kit (Sigma Aldrich, USA).

Serum samples : Serum samples analyzed in this study were obtained from 187 donor women on consent who visited the Sir Sundarlal Hospital, Banaras Hindu University, Banaras. The age of the volunteers ranged between 20-60 years. The volunteers had not undergone any prior screening for HPV infection or cervical abnormalities.

Positive sera : Sera from cancer patients (n=10) who also tested positive for HPV DNA of either 16, 18 and 45 genotypes in the DNA hybridization

assay (Linear array HPV Genotyping test Kit, Roche Molecular System, USA) were screened for L1 specific antibodies in an indirect ELISA coated with respective VLPs (VLP-based ELISA) as described elsewhere (12). The samples which showed high titres were further screened in the neutralization assay with the pseudovirions of the respective HPV genotypes (courtesy Dr. J.T. Schiller, NIH, USA) as described elsewhere (15). The samples showing significant neutralization titres in the assay were classified as positive sera that contained L1 specific antibodies of the respective genotypes. The HPV16 L1 human reference anti-serum (NIBSC, The UK) was also used as one of the positive sera in the 16 L1 specific GST capture ELISA.

Negative sera : Negative sera (n=20) were constituted by sera obtained from young adolescent girls that showed no reactivity either in the VLP-based ELISAs or the pseudovirion neutralization assays for HPV genotypes 16, 18 or 45.

Standardization of ELISA : Microtitre plates with matrices of different binding strengths for proteins, (low=Polysorp™, medium=-Medisorp™ and high=Maxisorp™) from Nunc, Denmark were evaluated for coating efficiency of casein–glutathione at a concentration of 200ng/well. Three different blocking agents- *viz*., casein hydrolysate (2%), fish gelatine (2%) and a commercial blocking reagent (Qiagen, USA) in phosphate buffered saline with 0.05% of Tween 20 (PBST) were studied for their blocking efficiency. Optimum concentration of capture antigen was determined by two fold serial dilution with the starting concentration of 400ng/well. Further, optimum concentration of binding antigen (GST-HPV16 L1) was determined by the addition of antigen in two-fold serial dilutions with a starting concentration of 2400ng/well. The bound antigen was probed with commercial HPV16 L1 specific monoclonal antibody (Camvir™; Novus Biologicals, USA). The optimal time of exposure to the chromogenic substrate-TMB/H_2O_2, was determined by incubating the assay plates for various time periods (10, 15, 20 and 30 min) prior to stopping the reaction with 1.25N H_2SO_4. The absorbance was measured at 450nm in an ELISA reader (Beckman Coulter DTX 880 Multimode detector USA).

GST capture ELISA : Ninety six well Maxisorp plates (Nunc Denmark) were coated with Glutathione casein (200ng/well) in 0.5M carbonate-bicarbonate buffer, pH 9.6. The wells were blocked with 2% (w/v) casein in phosphate buffer saline (PBS-casein) containing 0.05% Tween 20 (Sigma-Aldrich, USA) for 1 hour at 37°C. Post washing, binding antigen- GST-HPVL1 (16, 18, 31, 45 or 52), was added at concentration of 600ng/well to respective wells and plates were incubated 37°C for 1 h. Subsequently sera for analysis was added in two-fold serial dilutions (1:25 to 1:3200) along the rows of wells and incubated at 37°C for 1h. Bound antibodies were detected using anti-human IgG HRPO (Sigma-Aldrich, USA; at the dilution of 1:10K in 2% casein-PBST. Appropriate control wells *viz.,* non-specific antigenic control (GST-Bovine Papilloma virus L1 protein) and the assay control.

Method qualification: *Assay Precision:* The assays were repeated thrice on five different days. The inter-assay and intra-assay precision was determined as a function of the coefficient of variation (%CV) across assays.

Statistical analysis : *Cutoff values:* Mean absorbance (A_{450}) of negative sera plus 3 standard deviations was taken as the cut-off values for the determination of sero-positivity in the GST capture ELISA for HPV 16, 18 and 45 L1 (16). The initial cut-off values for HPV serotypes (31 and 52) were determined by frequency distributions (17). The final ELISA cut-off values were calculated using the Receiver Operator Curve (ROC) analysis (MedCal® V 11.5.1.1).

Determination of Sensitivity and Specificity: Sera characterized as positive and negative (20 samples each) as detailed above for the L1 antigens of genotypes HPV 16, HPV18 and HPV45 were assayed for the reactivity in VLP

based indirect ELISAs and GST capture ELISAs. The sensitivity and specificity were calculated using the 2 x 2 diagnostic test statistics (MedCal® V 11.5.1.1.) against the VLP ELISA readings as standard

Results

Purification of recombinant GST-L1 proteins : The affinity purified GST-L1s conformed to a size of ~82.0 kDa as visualized on SDS-PAGE profile (Fig.1a) and Western blot that was probed with anti-HPV L1 antibody (Fig. 1b). The additional product in the SDS-PAGE and western blot corresponds to the GST protein (Fig. 1c), most likely a cleavage artefact normally observed in recombinant expression of proteins with N-terminal fusion with GST.

ELISA Standardization : Maxisorp™ microtitre plates seemed to be the the most efficient binding matrix as indicated by the relatively higher A_{450} values even at higher dilutions of HPV 16 L1 specific antibody (Camvir™). The most efficient blocking was achieved with 2% casein in PBST. The optimum concentration of the casein-glutathione and GST-HPV L1 was 200ng/well and 600ng/well, respectively, as indicated by the A_{450} absorbance profile in the standardization trials (Fig. 2a and 2b). The optimum period of incubation with the chromogenic substrate (TMB/H_2O_2) was 10 min.

Statistical Analysis : The variability tests for the GST-capture assay of each of the HPV genotypes showed percent coefficient of variation CVs well below the limit set for precision and accuracy (Table 1). The results therefore indicate that the capture ELISAs meet the acceptance criterion prescribed for a validated immunoassay (17, 18, 19).

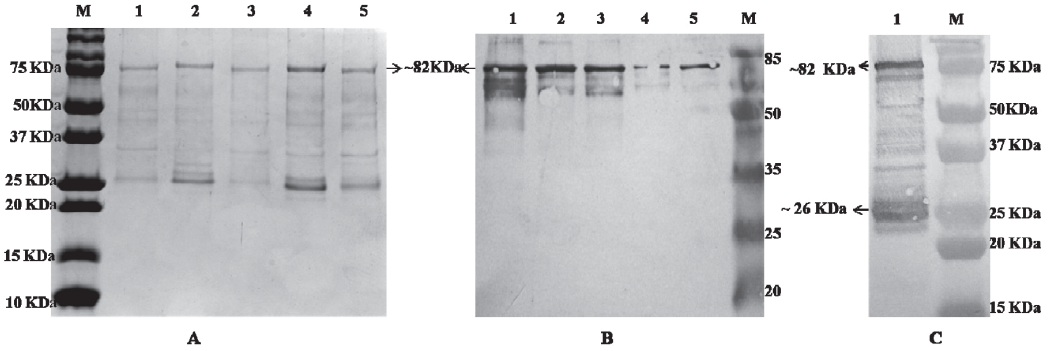

Fig. 1. Analysis of the affinity-purified recombinant GST-L1 protein
A: SDS PAGE and B: Western blot analysis of GST- L1 protein purified using Glutathione Sepharose® 4B affinity matrix. Lane 1- GST-16L1; Lane 2- GST-18L1; Lane 3- GST-31L1; Lane 4- GST-45L1; Lane 5- GST-52L1; Lane M- Pre-stained marker. C: Western blot analysis of HPV16 GST L1 probed using anti-GST antibody.

Table 1. Coefficient of variation (CV) of genotype specific GST-capture ELISAs.

Assay	%CV				
	GST-16 L1	GST-18 L1	GST-31L1	GST-45L1	GST-52L1
Inter-plate	6.05	3.02	8.44	9.10	6.53
Day-to-day	5.15	6.53	7.34	8.01	9.02
Inter-laboratory	8.61	9.63	11.02	9.03	8.04
Intra-laboratory	7.18	4.18	6.02	9.20	8.02

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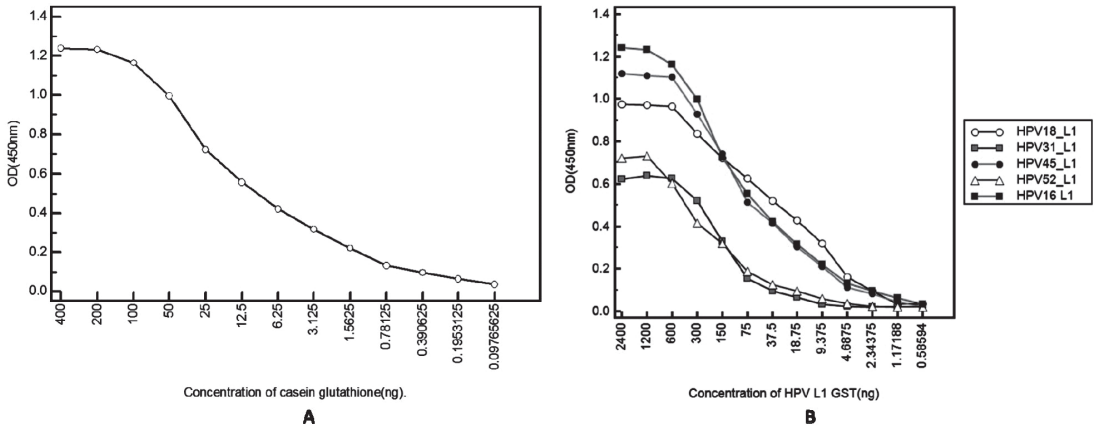

Fig. 2. Standardization of GST-capture ELISA.
A: Curve plotted as absorbance (A450) vs. concentration of coating agent (casein coupled with glutathione) performed with a two-fold serial dilution of coating agent (starting concentration; 400ng/well). The binding antigen (GST-16L1) was used at a constant concentration (600ng/well).
B: Curve plotted as absorbance (A450) vs. concentration of binding antigen (GST-16, 18, 31, 45, and 52L1) performed with a two-fold serial dilution of GST-L1 (starting concentration; 2400ng/well). The capturing antigen (casein-glutathione) was used at a constant concentration of 200ng/well. The commercial monoclonal antibody specific to HPV16 L1 (Camvir™) was used for detection of the bound antigen.

Seroprevalence : The cut-off values for the GST-capture ELISAs of L1 genotypes 16, 18, 31, 45 and 52 at 1:200 dilutions of sera were 0.473, 0.638, 0.329, 0.368 and 0.225 respectively. Out of 187 tested 80, 61, 31, 19 and 1 samples showed absorbance (A_{450}) above the cut-off values with GST-L1 capture ELISAs with HPV genotypes 45, 16, 18, 52 and 31 respectively (Fig. 3). There was significant cross-reactivity of the serum samples across genotypes (Fig. 4). All the sero-positive sera for HPV18L1 and the 52L1 reacted with one or the other genotype assayed. Only 4 sero-positive samples of 16L1 and 10 of the total 80 sero-positive samples of 45 L1 were specific to the respective genotypes.

The GST-capture ELISAs for the HPV genotypes 16, 18 and 45 showed sensitivity values of 94.7 %, 88.9% and 89.5% respectively. The specificity values for the ELISAs were 95.2%, 90.9% and 95.2 % respectively.

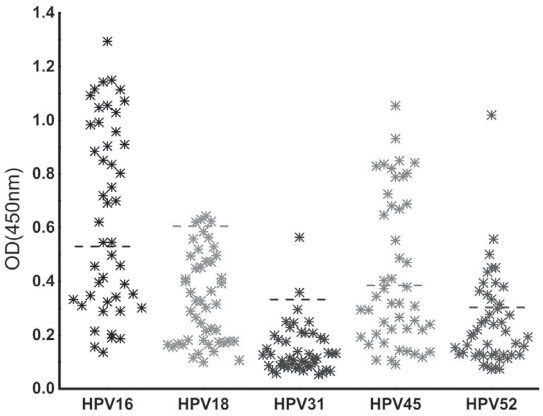

Fig. 3. Seroprevalence of antibodies to HPV L1 type 16, 18, 31, 45 and 52 in Indian women. (n=187). Dotted lines denotes the cut-off OD in the ELISA for the respective HPV types.

Discussion:

The majority of the estimated total deaths from cervical cancer, especially in the past few decades, have been reported to occur in the developing countries (20). The absence of systematic screening of women for cervical abnormalities is the primary cause for this skewed mortality rate in the less developed regions (20). Periodic and mandatory screening

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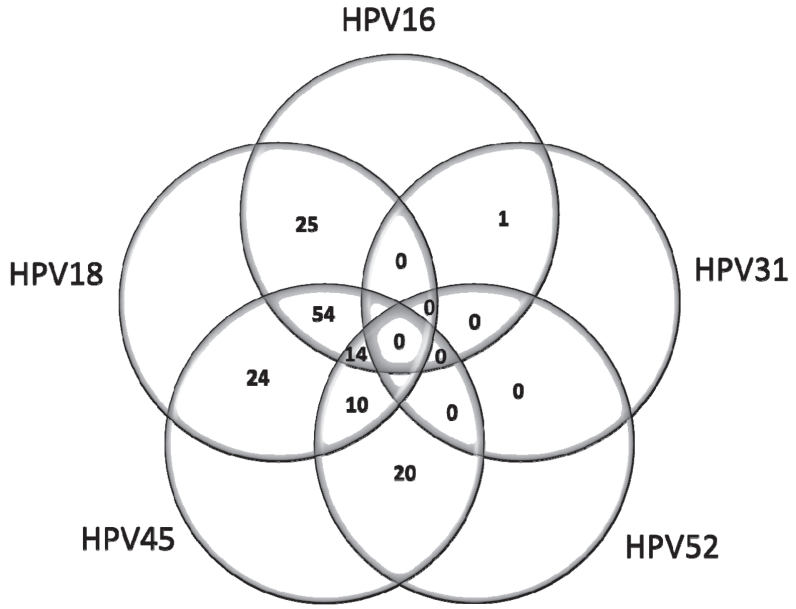

Fig. 4. Venn diagram depicting cross-reactivity of sero-positive samples across HPV genotypes. The number of samples showing sero-positivity to L1 of a specific HPV genotype is denoted in the corresponding set. The cross-reacting serum samples between HPV genotypes are denoted in the intersections of the respective sets.

of the large population of sexually active women in these regions with the current method of tissue-sampling (Papanicolaou test, Colposcopy, Biopsy etc.) is an expensive proposition. Besides, the clinical procedures involved, also require considerable skill and logistical resources that are seriously limiting in the nation states of these regions (21). Hence, a simple assay system that also holds promise in cost effectiveness such as the GST-capture ELISA, might just be the answer for large-scale screening of women in the less developed parts of the world.

The GST-capture ELISA first proposed by Sehr *et al.* 2001 (13) has been modified in this study only to the extent of using a purified form of the antigen than the crude recombinant *E. coli* lysates. We reckoned that a well characterized antigen is essential for the development of test kits and/or protocols that meet the precision and accuracy desired of a diagnostic assay (22). Further, the capture ELISA format was also evaluated for the determination of the seroprevalence of antibodies to the L1 of HPV genotypes 31, 45 and 52 besides the commonly studied 16 and 18 HPV types.

The yield of purified GST-L1 fusion proteins of the HPV genotypes ranged between 6.0mg-9.0mg/L of recombinant *E. coli* BL21 culture. Although modest, we reckon that the production costs would still be relatively less expensive than involved in the production of VLPs that need considerable downstream processing (23).

The GST-capture ELISA format was further standardized to establish a robust, reproducible assay protocol. The precision values (%CV) suggest that the ELISA meets the diagnostic assay criteria laid down by the International *Conference on Harmonization (*http://www.ich.org/; Quality Guidelines).

Seroprevalence studies in a population would invariably require established reactive and non-reactive sera (positive and negative control

sera) to determine for actual positive and negative serum samples in the ELISA. However, reference standards (except the HPV 16L1 human sera, NIBSC, the UK.) for the L1s of most HPV genotypes are not readily available. A characterized negative serum from a known, uninfected, individual is also difficult to obtain. We therefore designed a logical approach for the identification of non-reacting serum, and reacting serum from the available pool of human sera for use as the necessary negative and positive controls in the HPV 16, 18 and 45 specific GST-capture ELISAs. A combination of two established serological assays namely the pseudovirion neutralization assay (15) and the VLP-ELISA was used for this purpose. VLPs, which are identical in structure to the native virus, are used as antigens in the indirect HPV VLP-ELISA. Hence, VLP-ELISA is generally accepted as the "gold-standard" for the determination of virus specific antibodies (16). Further, the pseudovirion neutralization assay is a well-established *in vitro* assay for the determination of neutralizing antibody titres in a sample. The results of these two assays taken together, prospectively identifies genuinely positive sera; that which consists of L1 capsid specific antibodies. Sera that originally showed no reactivity in either the GST capture ELISA or the VLP ELISA, as was expected, did not show neutralizing titres in the pseudovirion assay and hence formed the panel of negative sera for the HPV 16, 18 and 45 specific GST capture ELISAs.

Lack of either VLPs or pseudovirions of HPV31 and 52 genotypes did not allow a similar approach to be followed for identification and classification of known control sera for assays specific to the two genotypes. Therefore the standard statistical methodology of determination of cut-off values by ROC was followed for the HPV 31 and 52 specific ELISAs in this study

The capture ELISAs were performed individually for each HPV genotypes (16, 18, 31, 45 and 52) to determine the serological status of the samples to the respective L1s. Surprisingly, most samples (42%) showed sero-positivity to HPV 45 (80 out of the 187 samples tested) followed by HPV 16 (32%), 18 (16.5%), and 52 (10%) respectively. There was only a single positive reactor for HPV 31. The results, except for the large number of positive reactors to HPV 45L1, generally followed the world-wide genotype specific prevalence pattern (24). There was significant cross-reactivity of sero-positive samples to L1 of one genotype to the L1s of other genotypes (Fig.4). Nearly, all samples sero-positive to the L1 of either 16 (61/187), 18 (31/187), or 52 (19/187) genotypes, also cross-reacted with the L1 of HPV 45. Only 12.5% (10 out of 80 sero-positive samples) were specific reactors to L1 of HPV 45. Similarly a minuscule 6% (4 out of 61 sero-positive samples) was specific to HPV 16 L1. All the sero-positive samples for 18L1 and 52L1 also reacted with the other genotypes, mainly either HPV45 L1 or 16L1. This prompted us to evaluate the sensitivity and specificity of the captures ELISA vis-à-vis the gold-standard assay, the VLP-ELISA. However, this could not be performed for the GST-capture ELISAs of HPV31 or 52 owing to the non-availability of genotype specific VLPs.

The determination of sensitivity and specificity was performed with the confirmed, genotype specific positive and negative sera for HPV16, 18 and 45 in the VLP-ELISA. However, the cross-reacting nature of the sera that were determined as positive reactors in the VLP-ELISA could not be ascertained due to the scarcity of VLPs. It is evident from the results that the GST-capture ELISA compares well with sensitivity and specificity *vis-à-vis* the VLP ELISA in determining sero-positivity and/or negativity of a sample to HPV L1. But the determination of genotype specificity of the antibodies in a sample by the GST-capture assay *vis-à-vis* the VLP-ELISA cannot be commented on with the limited data for the latter assay in this study.

A direct correlation between the cross-reactivity of sera to the homology of protein sequence of L1s among HPV genotypes could not be established. But the L1 capsid protein of the high-risk HPV genotypes share significant

homology in amino acid sequence (25). Hence, cross-reactivity is most likely, due to the presence of non-conformational antibodies to the linear epitopes of L1 (26).

The L1 proteins expressed in fusion with the GST are reported to be compromised in the ability to form VLPs but are shown to assemble in a pentameric structure where the GSTs occupy the periphery (27). The antigen in the GST-L1 pentameric form has the internal regions involved in the capsid assembly exposed. These regions are also fairly conserved across HPV genotypes and hence might contribute to the antibody cross-reactivity of the serum samples across genotypes. Another aspect worth consideration is the evaluation of the infection status of the volunteers involved in the study. Multiple infections with different HPV genotypes would in all probability influence the antibody repertoire of the host. At least the current infection status can be ascertained with the established HPV DNA testing methodologies (28).

Conclusion:

The study strengthens the case for application of GST-capture ELISA in preliminary screening of women for exposure to HPV. The comparable sensitivity and specificity of the capture ELISA to the current gold-standard (VLP-ELISA), the ease of antigen production, and the format of the assay (capture versus indirect), makes it a promising assay for large scale screening of individuals in low–resource settings. However, significant cross-reactivity of the positive reactors across genotypes was observed in this study. Therefore the application of the assay format for delineating the genotype specific humoral response, especially in studies to determine vaccine efficacy, would be limited.

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