A New Sandwich ELISA Test Simultaneously Detecting E7 Proteins of HPV-16, 18 and 45 in Cervical Smears

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Keywords: Human papillomaviruses; E7 Oncoprotein; Cervical cancer screening

Abbreviations: CIN: Cervical Intraepithelial Neoplasia; ELISA: Enzyme-Linked Immunosorbent Assay; hrHPV: High-risk Human Papillomavirus; HERMES: HEllenic Real-life Multicentric Cervical Screening; PIPAVIR: Persistent Infections by Human PapillomaViruses.

Introduction

Persistent infections by human papillomaviruses (HPV) are the main etiologic factor for cervical pre-cancer and cancer [1-3]. About forty HPV genotypes can infect epithelial squamous and glandular cells in the cervical mucosa. Based on epidemiological and biochemical data only a subgroup of HPV types, referred to as high-risk HPVs, is associated with high-grade intraepithelial lesions that have a high potential for progression to invasive carcinoma. Infections by high-risk HPV genotypes have been detected in virtually all cervical cancers [1]. At least 15 high-risk HPV types have been associated with this cancer. HPV-16, HPV-18, and HPV-45 are the most prevalent genotypes in cervical cancer [4], collectively accounting for approximately 75% of all cervical carcinoma worldwide [5]. After infection by high-risk HPV types the control of viral gene expression is tightly regulated but becomes deregulated during progression to more severe dysplasia [3].

This leads to deregulated transcription of the early viral genes E6 and E7, and to cell proliferation and genomic instability[6], often resulting in the integration of episomal HPV genomes into host chromosomes [7,8]. This results in increased expression of the viral genes E6 and E7 [9], and an increase of E7 protein levels during early carcinogenesis [10]. The consistent overexpression of these two oncogenes is necessary and sufficient to induce and maintain the transformed phenotype of cervical cancer cells [11-13]. Since immortalization of keratinocytes by the E7 oncoprotein involves its ability to bind and thereby functionally inactivate cell-cycle regulatory proteins such as the retinoblastoma tumor suppressor protein [10,14], the levels of E7 oncoproteins of carcinogenic HPV types may be specific markers for the detection of cervical pre-cancer and cancer.

Cytological assessment of cervical smears, by the Papanicolaou test [15,16], suffers from a high rate of false negative results, which might be improved by the introduction of molecular biomarkers. New technologies for cervical cancer screening are urgently needed. Previous studies suggest that high-risk HPV E7 proteins are regularly expressed in cervical carcinoma and in their high-grade precursor lesions, suggesting that high-risk E7 oncoproteins are necessary for this cancer and may serve as new tumor biomarkers [10,17-20]. In the present study, we developed and validated a new ELISA test capable for the simultaneous detection of the E7 proteins of HPV-16, HPV-18, and HPV-45.

Abstract

Persistent infections by high-risk papilloma viruses (HPV) are the main etiological factor for cervical cancer, and E7 oncoproteins were suggested as new markers for tumor progression. The objective of this study was to generate a new Enzyme-Linked Immunosorbent Assay (ELISA)-based detection system to monitor expression of the E7 proteins of the high-risk HPV (hrHPV) types HPV-16, HPV-18, and HPV-45 in cervical smears. Using a combination of rabbit monoclonal antibodies raised against E7 proteins of HPV-16 and HPV-18/HPV-45, respectively, a trivalent E7-ELISA was developed and validated, using recombinant E7 proteins of various HPV types and lysates from E7-positive cervical carcinoma cells. The amount of 0.5 picogram of E7 protein per well was determined as detection limit.

The E7-ELISA was used to determine E7 protein levels in cervical smears obtained from a total of 67 women. E7 protein concentration was below the detection limit in all HPV-negative smears, and E7 protein concentrations above background were found in some HPV-positive cervical samples. Together the work described herein provides a new tool for the simultaneous detection of E7 proteins of the three most prevalent in cervical neoplasia hrHPV types.
Material and Methods

Clinical samples

Nineteen cervicovaginal samples tested positive for HPV 16 or 18, and 48 samples tested negative for hrHPV were analysed for the present study (Table 1; extended clinical procedures are available in Supplementary File 1). 15 samples positive for HPV type 16 (n=12) or 18 (n=3) came from a cohort of women aged 25 to 55 years who participated in the “Hellenic Real-life Multicentric Cervical Screening” (HERMES) study [21]. Four samples positive for HPV type 16 (n=3) or 18 (n=1), as well as the 48 HPV negative samples, came from the PAPPARVIR (Persistent Infections with human Papilloma Viruses; www.pipavir.com) pre-study population. The classification of the cytology results was conducted according to the Bethesda 2001 directive [22]. All women samples were obtained in Thinprep PreservCyt Solution and stored at room temperature prior to E7 determination.

Characterization of RabMAbs and polyclonal goat antibodies by direct ELISA

E7 proteins of HPV types 11, 16, 18 and 45 were purified as described [23]. The generation of rabbit monoclonal antibodies (RabMAbs) 42-3 and 143-7, as well as polyclonal goat antisera to HPV-16 E7 (Goat 1) and HPV-18 E7 (Goat 2), was described previously [17,20]. Increasing amounts of the E7 proteins encoded by HPV-16, HPV-18, HPV-45 and HPV-11 (low risk) were added in 100 µl coating buffer (0.1 M NaHCO3, pH 9.6) to each well of a 96-well plate (Maxisorp F, Nunc, Vienna, Austria) and incubated overnight at 4°C. Wells were washed three times with washing buffer (0.05% Tween20/1x PBS, pH 7.4). Subsequently, 300 µl blocking buffer (Mikrogen, Neuried, Germany) containing increasing amounts of RabMAbs or goat polyclonal antibodies were added to each well, followed by an incubation for 1 h at room temperature. Wells were washed three times with washing buffer. 100 µl polyclonal HRP-conjugated secondary antibody (swine anti-rabbit antibody (0.3 µg/µl; Dako) or rabbit anti-goat antibody (0.5 µg/µl; Dako)) was added to each well and incubated for 1 h at room temperature. Wells were washed six times with washing buffer. 100 µl tetramethylbenzidine (TMB) detection reagent (Mikrogen GmbH, Neuried, Germany) were added followed by vortexing for 1 min. After washing the wells three times with washing buffer, 300 µl of blocking buffer were added to each well and incubated for 2 h at room temperature. Wells were washed three times with washing buffer followed by the application of recombinant protein, cell lysates or lysate from clinical samples, as appropriate. Subsequently wells were incubated for 1 h at room temperature. For detection of E7, a 1:1 mixture of biotinylated goat 1 and goat 2 antibodies diluted in diluent buffer was added to each well and incubated for 1 h at room temperature. Subsequently wells were washed 3 times with washing buffer and filled with 100 µl streptavidin-poly-horseradish peroxidase (poly-HRP) conjugate (PolyHPR40; SDT, Baesweiler, Germany) diluted with diluent buffer. Following an incubation of 1 h at room temperature, wells were washed six times with washing buffer. 100 µl of detection reagent were added to each well and incubated for 30 min in the dark at room temperature. The reaction was stopped by adding 100 µl stop solution, and absorbance was measured at 450 nm. E7 protein levels in cervical smears were calculated according to the standard curve obtained for recombinant E7 proteins.

Detection of HPV-16, 18 and 45 E7 by Sandwich ELISA

96 well plates (Maxisorp F, Nunc, Vienna, Austria) were coated with 100 µl coating buffer (0.1 M NaHCO3, pH 9.6) containing 0.25 µg RabMAb 42-3 and 0.25 µg RabMAb 143-7 and incubated overnight at 4°C. After washing the wells three times with washing buffer, 300 µl of blocking buffer were added to each well and incubated for 2 h at room temperature. Wells were washed three times with washing buffer followed by the application of recombinant protein, cell lysates or lysate from clinical samples, as appropriate. Subsequently wells were incubated for 1 h at room temperature. For detection of E7, a 1:1 mixture of biotinylated goat 1 and goat 2 antibodies diluted in diluent buffer was added to each well and incubated for 1 h at room temperature. Subsequently wells were washed 3 times with washing buffer and filled with 100 µl streptavidin-poly-horseradish peroxidase (poly-HRP) conjugate (PolyHPR40; SDT, Baesweiler, Germany) diluted with diluent buffer. Following an incubation of 1 h at room temperature, wells were washed six times with washing buffer. 100 µl of detection reagent were added to each well and incubated for 30 min in the dark at room temperature. The reaction was stopped by adding 100 µl stop solution, and absorbance was measured at 450 nm. E7 protein levels in cervical smears were calculated according to the standard curve obtained for recombinant E7 proteins.

Results and Discussion

Characterization of anti-E7 antibodies

To control the quality of antibodies selected for this study, direct ELISA experiments were performed. ELISA plates were coated with increasing concentrations of recombinant E7 proteins, and incubated with increasing amounts of purified RabMAbs 42-3 [17] and 143-7 [20], respectively. The bound rabbit monoclonal antibodies were detected by incubation with HRP-conjugated anti-rabbit-antibodies, incubated with substrate and quantitated by a Plate reader. Starting at an E7 protein concentration of 500 pg per well, OD values over the background could be obtained. For both RabMAbs, signal saturation was reached at a concentration of 50 ng E7 protein per well and 81 ng RabMAb (Figure 1A), indicating that the interaction between both RabMAbs and their cognate antigens is similar in quantitative terms (Figure 1A). We also tested the interaction of affinity-purified goat polyclonal antibodies raised against HPV-16 E7 (goat 1) and HPV-18 E7 (goat 2) with their cognate antigens. We found that signals over background were obtained with 10 ng of HPV-16 E7 for goat 1 and 10

Citation: Metzger C, Pittl A, Kaufmann AM, Agorastos T, Chatzistamatiou K, et al. (2016) A New Sandwich ELISA Test Simultaneously Detecting E7 Proteins of HPV-16, 18 and 45 in Cervical Smears. Clin Microbiol 5: 260. doi:10.4172/2327-5073.1000260
ng of either HPV-18 E7 or HPV-45 E7 with goat 2. Both goat antibodies displayed a weak but detectable cross reactivity with low-risk E7 proteins, as shown here for the E7 protein of HPV-11 (Figure 1B).

**Figure 1**: Characterization of E7 antibodies. ELISA plates were coated with increasing amounts (0.025-250 ng) of recombinant E7 proteins, as indicated, and incubated with (A) increasing amounts (1-243 ng) of a 1:1 mixture of RabMAbs 42-3 and 143-7, followed by a secondary polyclonal swine anti-rabbit antibody coupled to HRP, and (B) increasing amounts (10-100 ng) of antibodies Goat 1 and Goat 2, as indicated, followed by a secondary anti-goat antibody coupled to HRP. Absorbance at 450 nm was measured after incubation with TMB.

**Development of an E7 sandwich ELISA**

Based on the results of the direct ELISA assays, a sandwich ELISA was established by coating ELISA plates with a 1:1 mixture of RabMAbs 42-3 and 143-7. For detection of bound E7 proteins, a 1:1 mixture of biotinylated antisera goat 1 and goat 2 was used. This setting was then tested with an increasing concentration of the E7 proteins of HPV-16, HPV-18 and HPV-45. For HPV-16, HPV-18 and HPV-45, signal over background was obtained starting at a concentration of 500 fg per well, and a steady increase of the signal was observed up to 10 pg of each E7 protein (Figure 2A). In the same setting, the addition of 100 pg of the low-risk E7 protein of HPV-11 gave no detectable signal. Multiple runs of the titration curves shown in Figure 2A were performed and used to determine the intra-assay and inter-assay variance of the ELISA (Table 1), which was found to be below 10% in all cases.

|               | HPV-16 E7 | HPV-18 E7 | HPV-45 E7 |
|---------------|-----------|-----------|-----------|
| Low range     | 4/8       | 3/7       | 4/6       |
| Medium range  | 5/9       | 3/6       | 3/6       |
| High range    | 4/7       | 6/7       | 4/5       |

**Table 1**: Intra- and inter-assay variance (%) of the E7 ELISA with recombinant E7 proteins of HPV types 16, 18 and 45.

To test the suitability of the sandwich ELISA for detection of endogenous E7 proteins present in cervical carcinoma cells, CaSki, SiHa (both HPV-16 positive), HeLa (HPV-18 positive) and MS-751 (HPV-45 positive) cells were grown and fixed in ThinPrep solution. The E7 protein content in extracts obtained from 20,000 and 50,000 cells each was determined by E7 ELISA, with signals over background being achieved with 20,000 cells per well in each case (Figure 2B).
Quantification of E7 proteins by sandwich ELISA in cervical smears

For initial clinical assessment of the sandwich ELISA described here, a set of cervical smears from 67 women (obtained from the HERMES study and the PIPAVIR pre-study; Table S1) was analysed. Cervical smears from women were taken up in ThinPrep solution according to standard procedures and characterized by cytology. HPV DNA was determined by HPV DNA genotyping. We found that the set of clinical samples contained 15 smears which were positive for HPV-16 DNA and 4 smears positive for HPV-18 DNA, plus 48 smears in which no HPV DNA could be detected. Cellular material from ThinPrep samples was lysed and E7 concentration determined by E7 sandwich ELISA, based on the calibration curve obtained with recombinant E7 proteins before (Figure 2A). E7 protein expression was undetectable in all HPV-negative samples, indicating that endogenous proteins from patient tissue do not give rise to nonspecific background.

In the HPV-16 DNA positive samples and the HPV-18 DNA positive samples, concentration of the respective E7 concentration varied between 0 and 4 pg per well (Figure 3). Unfortunately, the unavailability of HPV-45 positive samples precluded the validation of the test for HPV-45 E7 in clinical samples. Of note, the sandwich ELISA described here provides virtually no background signals for E7 proteins of HPV-16 in clinical samples. Background problems prevented previous attempts to establish a sensitive sandwich ELISA for HPV-16 E7 (our unpublished results). Probably, most available antibodies to HPV-16 E7 cross-react with epitopes present elsewhere in the human proteome. Reduction of background signals in this particular case is probably achieved by the fact that RabMab42-3, used here as coating antibody, recognizes in the Zn finger region of HPV-16 E7 protein a conformational epitope, which apparently is not represented in the cellular proteome [17].

**Figure 2:** Setup and technical validation of a sandwich ELISA for the simultaneous detection of HPV–16, -18, -45 E7 proteins: ELISA plates were coated with a 1:1 mixture of monoclonal RabMAb 42-3 and 143-7. (A) Increasing amounts (0.5-100 pg) of recombinant HPV-E7 proteins were added as indicated, (B) protein extracts of CaSki, SiHa, HeLa, and MS751 cells prepared from 20,000 and 50,000 cells were added as indicated. Subsequently, a 1:1 mixture of biotinylated antibodies Goat 1 and Goat 2 was added, followed by the addition of SA-polyHRP40. After incubation with TMB, absorbance at 450 nm was measured. OD values were converted to E7 protein concentration according to the standard curve. HPV-11 E7 protein and lysis buffer were used as negative controls.

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Antibodies to E7 proteins of high-risk HPV types have been used previously to assess a potential role of these proteins in cervical cancer. On the one hand, sandwich ELISAs were described for the separate detection of the E7 proteins of HPV-16 and HPV-18, respectively, with a reported detection limit of one hundred picogram of E7 proteins [24]. Along the same lines, we have reported previously that 500 fg of E7 protein of HPV-18 can be detected in a sandwich ELISA using goat serum 2 as coating antibody and biotinylated RabMab 147-3 as detection antibody [24]. On the other hand, several groups have used recombinant E7 proteins to set up ELISAs that allow detection of antibodies against E6 and E7 proteins of human papillomavirus types 16 and 18 in patient serum [25], thereby providing an alternative scenario to evaluate the role of E7 (and E6) proteins in cervical carcinogenesis.

The work described here establishes for the first time a sandwich ELISA suitable for the simultaneous detection of the E7 oncoproteins of HPV types 16, 18, and 45. Of note, reliable detection of HPV-16 E7 in clinical samples was not achieved in previous studies using polyclonal goat serum (goat1) as coating antibody and biotinylated RabMab (RabMab 42-3) as detection antibody. This ELISA format revealed sporadic background signals which precluded its use with clinical samples (our unpublished data). In this communication, the problem was overcome by instead using a mixture of both RabMabs (42-3 and 143-7) as coating antibodies, and achieving detection with a mixture of affinity-purified and subsequently biotinylated polyclonal goat sera (goat1 and goat2). It is shown here that the current assay detects recombinant E7 proteins of these three HPV types with a detection limit of 500 fg and has no reactivity towards E7 protein of the low risk HPV type 11. The assay can be performed from cervical swabs stored in ThinPrep buffer, providing compatibility with current clinical procedures. Using this assay, we demonstrated that signals for E7 proteins were undetectable in smears derived from HPV DNA negative women, whereas E7 levels over background were obtained in some of the HPV DNA positive women.

The very low number of samples from patients with histologically confirmed cervical intraepithelial neoplasia is the main obstacle in our study, in order to verify the clinical utility of the E7 ELISA test, especially in the framework of triaging women with HPV DNA positive results or abnormal cytology. We provide however data showing that this new diagnostic molecular test is worthy to be evaluated in large studies with sufficient number of cases in the different subgroups, in order to determine its clinical performance.

**Conclusion**

A trivalent E7-ELISA was developed and validated, specifically detecting E7 proteins of HPV-16 and HPV-18/HPV-45, with a detection limit of 0.5 picogram. In cervical smears, E7 protein concentration was undetectable in all HPV-negative samples, and E7 protein concentrations above background were found in some HPV-positive samples. In summary, we provide here a new tool for the simultaneous detection of E7 proteins of the most prevalent in cervical neoplasia hrHPV types.

**Acknowledgement**

For expert technical support, we thank Ursula Schiller and Bianca Mandt.

**Authors’ contributions**

HP, CM, AP, and IK developed and technically validated the trivalent E7 sandwich ELISA. CM, AP, and IK prepared lysates and tested them by E7-ELISA. AMK, TA, and KC provided clinical samples and information on their HPV status. IK, HP, WZ and PJ conceived...
the study and drafted the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Funding

Work in the laboratories of P.J., A.M.K., T.A., and O.B. was supported by the European Union through FP 7 project PIPAVIR (Project No.304927). P.J. also acknowledges funding by the County of Tyrol (Zervixkarzinom, Project No. WIF-273-01-00003/01-0150). The cobas HPV test kits and consumables for the HERMES study were provided by Roche Diagnostics (http://molecular.roche.com/).

Conflict of interest

I.K. and O.B. are employees of Mikrogen GmbH. P.J., H.P., and W.Z. are listed as inventors on a patent application relating to the E7 ELISA. AMK, TA, CK, AP, and CM declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval was obtained from all individual participants included in the study. This article does not contain any studies with animals performed by any of the authors.

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