New molecular diagnostic approach of human papillomavirus detection: the role of experimental design in the development, analytical and clinical evaluation

Doctoral (Ph.D.) thesis

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INTRODUCTION

Experimental design is widely used in science; however some special implementation would greatly help research in that particular area. The original goal of the Taguchi method was to find the optimum conditions and to estimate its numerical parameters (Taguchi, 1993). However, the Taguchi system contains much more information because it could calculate the numerical parameters not only for the optimum conditions but for any combinations of the factors. An advantage of the method is that the optimization and the determination of the analytical parameters of the assay can be carried out in one step. Furthermore, the recalculation of these parameters can be carried out, which is impossible in the traditional methods. The developed method was applied generally to determine the multiparametric optimization of different analytical systems, e.g. ELISA method to detect ScFv phages and multiplex PCR methods to detect human papillomaviruses.

Considering the role of HPVs in malignant lesions (zur Hausen et al., 1975), the accurate and high throughput detection and genotyping of HPVs is of great importance. The diversity of the HPV spectrum and the high incidence of multiple infections make it necessary to establish reliable methods for identification of the various HPV genotypes, not only for epidemiologic studies, but also for patient management.

DNA based methods (PCR and hybridization) are widely used to detect of HPV infections.

A method described by Yoshikawa et al. (Yoshikawa et al., 1991) employs an L1–based PCR system consisting of two consensus primers and a restriction fragment length polymorphism analyses (RFLP). This PCR system is not sensitive enough for many clinically important genotypes (there are even important genotypes which are not amplified or amplified at a very low sensitivity, e.g. HPV 16), it is time-consuming, and not a feasible method for HPV genotyping high number of clinical samples. A new method was devised to

address the unmet requirements of the clinical detection of human papillomavirus.
To evaluate the performance of this new HPV detection method clinical studies and comparisons were carried out.

AIMS OF THIS THESIS

By the introduction of new experimental design methods in the development of new human diagnostic devices makes possible to develop methods involving difficult optimization problems. The aims of this work were to demonstrate the application of Taguchi optimization by the examples of an ELISA and a PCR method. With the improved method one would able to directly estimate several parameters of an ELISA system: the calibration curve, the sensitivity of the optimum assay, the intra-assay and inter-assay variability. Further application of this method devised to find the optimum primerset concentration for a highly multiplex PCR system detecting human papillomavirus, with the following features: it detects a broader spectrum of HPV genotypes, is dynamic to recognize newly emerging HPV types, yields a balanced amplification and features an automatizable genotyping method (hybridization). The carefully selected amplicon is based on Yoshikawa L1 (Yoshikawa et al., 1991) amplicon. The comparison of the system to other systems, to access its analytical characterization analytical evaluation is needed to be carried out. To access clinical behavior of the system a clinical evaluation of the system is devised. As a part of the clinical evaluation the pelvic inflammatory disease proposed to be studied as a predictor of cervical cancer.

MATERIALS AND METHODS

HPV controls.

We subcloned the following HPV genotypes: 2a, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55/44, 57, 58, 59, 61, 66, 67, 68, 72, 73 into the pCR2.1 Topo vector. Plasmids contained subgenomic fragments of the HPV genomes which embody the L1F/L1R1 amplicons. Clinical samples were used in case of other sequencing verified genotypes (HPV types 30, 43, 56, 70, 74, 82, 84, 89, 90, 91).

Polymerase Chain Reaction protocols

Our improved PCR protocol using the L1F/L1R primers was the following: the amplification reaction contained a mixture of 3.4 μM L1F and 3.6 μM L1R primer set, 2.5 μl 10× PCR Gold buffer, 1 unit of AmpliTaq Gold, 2 mM of MgCl2 solution and 250 μM of each dNTP. The amplification profile was 95°C for 10 minutes, 10 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 72°C for 30 seconds, 35 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds, and terminal extension 72°C for 4 minutes. The reaction volume was 25 μl containing 5 μl DNA sample. PCR products were subjected to electrophoresis followed by hybridization.

Sequencing

PCR products were treated by the High Pure PCR Product Purification Kit. This template was then sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1. Sequencing reaction products were run on an ABI Prism 310 Genetic Analyzer. The DNA sequences were aligned with the public database on NCBI homepage using BLAST algorithm.

Hybridization probes

The probes used for solid-phased hybridization were fluorescein-labelled in case of genus and genotype specific oligonucleotides, and digitoxigenine-labelled in case of HPV-Internal Control specific oligonucleotides. All labelled nucleotides were HPLC purified.

HPV detection and typing by solid-phased hybridization

The biotinilated PCR products (products of amplification by one labelled primer) are captured onto the surface of a streptavidin-coated microplate well. The complementary chain is eluted from the immobilized PCR product and the hybridization is carried out in the presence of specific fluorescein- (in case of genus and genotype specific probes) and digitoxigenin- (in case of internal control specific probe) labelled probes. The bound probes are reacted with anti-fluorescein-HRPO (HRPO-horse-radish peroxidase) and anti-DIG-AP (AP-alkaline phosphatase) antibody. Substrate development is done sequentially (separately). The HRPO substrate yields fluorescent signals measured at ex/em: 324/410, while the substrate of AP yields fluorescein signals measured at ex/em: 355/460.

Sample acquisition and preparation

Cervical specimens were collected by cervical brush samplers and transported in PBS from STD (sexually transmitted disease) outpatient clinics. The samples were processed by centrifugation (2000 g, 10 minutes), the pellet was washed in PBS, lysed in 250 µl lysis solution containing HPV internal controls (0.5 mg/ml proteinase K, 0.01 M TRIS-HCl pH8, 0.001 M EDTA pH8). The samples were then vortexed and incubated for 30 minutes at 56°C. The following DNA isolation steps were carried out on a TECAN RSP150 robot using silica binding chemistry.

Comparison of L1F/L1R system and the Hybrid Capture II test

Cervical samples issued as ASCUS (Atypical Squamous Cells of Undetermined Significance) or higher in prior cytological examinations were analyzed using the Hybrid Capture II test (HC II) and the L1F/L1R system, following the instructions of the manufacturers. The samples included in this study were collected by Digene Cervical Sampler and were split: one part was used for Hybrid Capture II detection, the other part was subjected to DNA extraction by High Pure PCR Template Preparation Kit. The amplicons of discordant results were sequenced in order to establish the true high-risk positivity/negativity.

Testing the system on clinical samples

Clinical samples issued as ASCUS or higher from a cohort of 360 persons were tested by the L1F/L1R system. Numbers of positive and negative cases were recorded, together with the prevalence of the different high-risk HPV types. Percentages from prevalence of 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) were compared to those published by Coutlee et al. (Coutlee et al., 2002).

Antigens and antibodies

Phages were prepared by transforming SURE E. coli cells with a ScFv phagemid pCANTAB5 from .Pharmacia, Uppsala, Sweden containing a rearranged adenovirus hexon monoclonal antibody Mab gene (Ruzsics et al., 1994) and rescued with M13KO7 helper phage. The preparation of phages followed the protocols described in the Pharmacia Recombinant Phage Antibody System Expression Module kit. The anti-M13-phage polyclonal serum were raised using purified phages as antigen as previously described Cooper and Paterson (Cooper and Paterson, 1991).The serum was purified by ammonium sulphate precipitation and cation exchange chromatography:

particulate material being removed by ultracentrifugation (Andrew, 1991). The biotinylated anti-rabbit antibody donkey was from Amersham Buckinghamshire, UK and Extravidin-HRPO was from Sigma Deisenhofen, .Germany.

ELISA for detection of ScFv phages

All reagents were prepared in PBS and the concentrations and conditions were changed according to the experimental layout forced by the Taguchi array. All incubations were carried out at 37^{o}C. Every condition was tested in triplicate. For coating of the wells the dilutions of the phages 12 600, 6300, 630, 6.3, 0.063, 0.0063 ng/ml were prepared in PBS. The protein concentrations were measured by the Coomassie binding method.

After overnight incubation, the coated wells were washed four times. The rabbit anti-phage polyclonal antibody was used as primary reagent dilutions: 1:5000, 1:10000 or 1:20000 for 1 h. The wells were washed four times and were either blocked for 30 min or were further processed without blocking according to the experimental layout. Biotinilated anti-rabbit donkey antibody was used as secondary reagent for 1 h dilutions: 1:500, 1:1000, 1:2000 . Again, a four- fold wash stage with or without blocking was used. Extravidin-HRPO conjugate was added 1:500, .1:1000, 1:2000 to detect the biotin label and it was washed four times without blocking. To detect the enzyme label, OPD substrate was used and the reaction was stopped after a period of time 5, 8, 12 .min by the addition of 4 N sulfuric acid. The plates were measured at 492 nm with background subtraction at 620 nm. Parallel control experiments were run omitting the coat but using the Taguchi layout.

Taguchi method

Calculations are performed with Microsoft Excel spreadsheet macro written in our laboratory. All the experiments were carried out in triplicate. The Taguchi experiments followed an experimental layout called the L18 array. We chose the L18 array because it has a few

advantages over the others in this particular application. For complete optimization the protocol consists of only 18 parallel setups in one experiment and is also characterized by a specific feature suppressing the interaction effects which otherwise can bias the calculation of optimum conditions. The latter is important because the interaction of factors is very common in ELISA. One modification used with the original L18 array is a new six-level factor A combining the first two original factors of the array assigned to the concentration of the coated material. The levels of this factor should be set so as to cover at least four magnitudes in concentration to serve as the basis for calculating the calibration curve. Other factors were assigned to conditions which should be optimized to give better performance in the assay. The last factor was not assigned and was used as an error factor factor e. The biochemical parameters were calculated as follows:

i. sensitivity: adding standard error twice to the lower infinite value of the logistic curve at the estimation of this value the standard deviation of the 18 experiments .was calculated and the largest was chosen for use;
ii. intra-assay variability: the average of the coefficient of variation of the triplicates of the 18 experiments;
iii. inter-assay variability: the coefficient of variation of the triplicate averages of the 18 experiments plus the intra-assay variability.

One-step-at-a-time method

All factors were kept constant except for one and the first observation was taken at the third level of that factor. The second observation was taken at the second level. If the response on the second observation was larger, a third observation was taken at the first level. Finally, keeping the largest response level of that factor constant a new factor was chosen and the procedure was repeated in the same manner.

Taguchi optimization for mapping the optimum concentration of the primersets

The factors, which are groups of primers, are according to the phylogenetic tree determined by ClustalW. The PCR and the hybridization was carried out as described above, samples are dilutions of plasmids of the cloned genotypes (100, 10, 1 copies/ul). The evaluation was a comparison of the sensitivity (detecting lower amount of target or with higher signal) between the benchmark (L1F/L1R) reaction (experiment 1) and the others. The signals are calculated as fold signals over the corresponding genotypes specific NTC signals.

Study to access the pelvic inflammatory disease as risk factor for cervical cancer

The study population consisted of two groups of women. The 2,215 patients with signs and symptoms of PID will be referred to as Group 1, they were enrolled during 1999-2001. Diagnosis of PID was based on physical, clinical and laboratory findings. Group 2 consisted of 4,217 women who attended gynecological visit in 2000, patients with signs or symptoms of PID were excluded from this group. HPV was detected according to the protocols above.

RESULTS

Taguchi optimization of the parameters of ELISA

To test the Taguchi method in the optimization of ELISA an antigen detection problem was chosen that had proven to be very troublesome, i.e., detecting antigen bound ScFv phages with polyclonal antibodies. Dealing with the ELISA of ScFv phages we found high backgrounds and low sensitivity hampering our efforts to find antigen specific phage clones. To circumvent this problem, a new anti-phage serum was raised in rabbits and we faced a multi-variable optimization of the ELISA assay. One set of Taguchi

experiments was carried out using phage coated wells and a parallel set of wells without antigen coat. The corresponding background was subtracted and the ‘ Larger-the-better’ transformation was used.
The calculated parameters display an ED50 of 55.80 ng/ml logistic curve parameters: a = 0.37, b = .0.68, c = 55.80, d = 1.54 and 1.47 ng/ml sensitivity (Chard, 1997), an inter-assay coefficient of variation of 6.35% and an intra-assay coefficient of variation of 4.06%. To check out the prediction we performed the one-factor-at-a-time method to find .the optimum condition (Anderson and McLean, 1974) which resulted in a very similar set of optimum conditions. The only difference between the two method results was the concentration of Extravidin peroxidase conjugate and the extra blocking step factor E , the effects of these factors are non-contributory as judged by the ANOVA table and for this reason too close to apply the method of one-factor-at-a-time. The two curves’ ED50 and slope were not statistically different $p > 0.05$ $F[1,18] = 0.001618$ for ED50 and $F[1,18] = 0.000742$ for slope.

Estimation of the ELISA assay parameters at the optimum levels of the factors

To access not only the optimum conditions but also more useful analytical parameters of the assay; we have taken advantage of the proposed special assignment with six levels of coated antigen concentration. Using the result of the Taguchi optimizations, based on this six-level factor, the standard curve of the optimum conditions can be calculated. Taking the optimum levels of the contributory factors, and using each level of factor A, the corresponding estimated results can be calculated and, using them, the calibration curve can be constructed. The other important consideration is the determination of the factors which can reduce the background but not contribute significantly to the performance of the assay. To pinpoint these factors we again used the original Taguchi experimental results of non-coated background wells and their factor contributions were compared to the original factor contributions. Factors with a

significant %V e value contribute to the background and if we use the lowest levels in bold we reduce the background and remove some of the confounding interactions making the estimations better. These considerations and the treatment of the data cannot remove all the effects of the interactions but the calculation after the data treatment gives a very close estimation of the calibration curve. The analytical parameters of the fitted curve agreed very closely with the estimated values parameters for A: a = 0.15, b = 0.68, c = 145.91, d = 1.27 for B: a = 0.37, b = 0.68, c = 55.80, d = 1.54 and according to the chi-squared test the two sets of points were similarly distributed with a probability of 0.99. Nevertheless, the calculated and the determined sensitivities were fairly close 7.22 ng/ml and 5.57 ng/ml, respectively. Finally, the inter-assay and the intra- assay variability estimates were 7.02% and 4.66%, respectively, compared to 6.35% and 4.06%. The results confirmed the predicted values suggest that the method is effective in the estimation of these parameters.
The Taguchi optimization of this pilot experiment established its further use, during the development of the L1F/L1R primer system and optimization of the application of DNA ELISA for the detection of the L1F/L1R amplicons this experimental design method proved to be very successful.

Development of the L1F/L1R primers.

During the development we first determined the L1C-PCR sensitivity spectrum over 46 HPV genotypes. On the basis of these results and the sequence data of the primer binding regions of the theoretically amplifiable genotypes, we designed 15 forward and 16 reverse primers. The priming efficiency of the primer set was optimized by the relative concentration of the primers and also by the design of the primer sequences. Introducing mismatches could help to reduce internal competition of the primers, but mismatches are kept at a minimum especially at the 3’ end of the primers. This approach resulted in a smooth priming efficiency distribution. The optimized reaction has very high sensitivities and uniform amplification power.

Notably the reaction also has very broad spectrum over the genotypes.
The L1F/L1R system detects theoretically 52 genotypes, from which the amplification and detection capabilities of 46 genotypes were tested in this study. Further studies are needed to provide data about HPV 69, 81, 83(MM7), 85 and the novel genotypes HPV 86, 87 (Menzo et al., 2001).
After the initial design, we evaluated the possible experimental space to find better solution considering the relative concentration of the phylogenetic groups of primers. Using the clustalw algorithm we constructed the corresponding phylogenetic trees for both the forward and the reverse primer sets.
In the next step the optimization experiments were carried out to access the optimum conditions to achieve the highest sensitivity over several genotypes. We used the Taguchi design to set up the experiment, however because the Taguchi optimization calculations are based on the assumption of the low factor interactions, and this requirements is no realistic in our system (see above) we used a slightly modified evaluation of the method. We carried out the experiments devised by the method, however we evaluated the results directly from these experiments, choosing the best experiment as an optimum. The Taguchi design uses orthogonal matrixes to separate the experiments evenly in the experimental space, by this way effectively sampling the possible experimental space. In several other cases we have found (data no shown) that this method is sensitive to find both local and global optimums of the systems in question. Both local and global optimum solution could be further mapped by setting up the factors around of the found optimum. However, usually only one step of optimization is needed to find a satisfactory solution.
According to the results there is a slightly better solution than the benchmark setup, with reduced amount of primers in several primer groups. It was discovered in other systems (at low multiplicity) that the primer concentration is usually have a concentration optimum

(Linz et al., 1990), however in such highly multiplex primers sets this is the first proof.

Evaluation of amplification

We compared the Yoshikawa system, using the original reaction conditions as described (Yoshikawa et al., 1991), to our L1F/L1R system. We tested the clinically important HPV genotypes in dilution series (HPV low-risk: 6, 11, 42, 55/44, HPV high-risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 66, 68) in order to compare the differences in sensitivity. When we compared the lowest detectable copy numbers/reaction, the L1F/L1R system proved to be more sensitive with orders of magnitude.

Both systems were evaluated to assess the genotype spectrum of the reactions. This latter was carried out detecting 46 genotypes at high copy number. The Yoshikawa system could not amplify or poorly amplified a number of HPV genotypes (e.g. 2a, 3, 7, 13, 27, 29, 35, 40, 42, 43, 55/44, 57, 73, 74, 84), while the L1F/L1R had a well balanced and much more sensitive amplification compared to the L1C-PCR.

The Yoshikawa PCR system showed a significantly lower sensitivity for the low-risk genotypes generally e.g. HPV 11, HPV 42, HPV 44/55 and for some high-risk genotypes (HPV 16, HPV 35), these could be detected only at very high copy numbers which is clinically unacceptable. Considering that clinical samples could contain only a small number of HPV infected cells, the general level of sensitivity of the L1C-PCR was also too low.

Specificity of the amplification was tested on several genital pathogenic agents, such as: Herpes simplex virus, Chlamydia trachomatis, Ureaplasma urealyticum, Neisseria gonorrhoeae, and Mycoplasma genitalium. The L1F/L1R system showed amplification only for HPV.

Genus-specific detection

For clinical applications it is advantageous if general detection of HPV amplicon is also possible. The amplification systems frequently cover a much broader genotype range than the detection systems, so the uncovered genotypes could be detected only by general detection of the amplicon. This helps to avoid the technically false negative results, moreover the detection of these genotypes might be clinically important. The general detection and genotyping of the same amplicon must produce concordant results, which virtually eliminates detection errors.

The L1F/L1R amplicon contains a conservative region that allows designing genus-specific probes. Using sequence alignments we devised a probe mixture containing 16 oligonucleotide probes.

Genotype- specific detection

The probes were designed for a unique highly diverse region, which is nested by conserved regions. Several micro- insertions and/or deletions improved the diversity of the regions. This allows highly specific probe design for each genotype. Chan et al. (Chan et al., 1995) compared a 291 bp conserved region from a 460 bp region of the L1 gene amplified by the MY09-MY11 primer pair in order to show phylogenetic relationship among 95 HPV types. Based on this data we chose the HPV types 6, 11, 44 from the Group A10 in order to test the performances of our hybridization system. We used these genotypes because they are highly similar to each other (Chan et al., 1995). To test the discrimination between these closely related genotypes we amplified a serial dilution of these genotypes with a sample containing internal control DNA and the tested DNA. Each PCR product was hybridized with the HPV 6, 11, 44 type specific oligos. Crosshybridization levels were assessed by comparing the hybridization signals with matching and not matching oligos. All genotypes discriminated from the others, e.g. the 5 mismatches that occur between the HPV 6 DNA and the HPV 11 probe were discriminated perfectly even in case of high copy number samples. Based on these experiments we generally applied this minimum 5

base mismatch rule during the design of the probes as criteria to design specific probes.
Sequences containing the L1F/L1R amplicons of the theoretically amplified 52 HPV genotypes were aligned to determine the specificity of type-specific oligonucleotide probes. Except the very closely related (Menzo et al., 2001) low-risk genotypes HPV 44 (Lorincz et al., 1992)and HPV 55 (Favre et al., 1990), all genotype-specific oligonucleotide probes contained five or more mismatches with the probe-binding region.

Clinical evaluation

Clinical performances of our system and the Hybrid Capture II test were compared on a set of 81 samples that have been issued as ASCUS or higher. The study design was to compare the detection capabilities of several clinically relevant, high-risk genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in both systems. In the cases of conflicting classification sequencing was carried out to clarify discordant results. Additionally we genotyped the low-risk and the high-risk samples.
After the first round of analyses we found concordant positive samples for both systems in 28 cases, while 40 samples were concordant negative. There were 6 samples which were L1F/L1R positive- HCII negative, and 7 samples L1F/L1R negative- HCII positive.
There was a good agreement of 83.9% (68 of 81 samples) between the two systems (kappa value=0,67, McNemar's chi square $P<0,0001$; $Q=0.65$, Yule test).
After this first step samples showing discordant results were sequenced in order to establish true high-risk positivity/negativity. Analysis showed that in 10 of 13 discordant cases L1F/L1R had indicated the correct results, 3 of 13 discordant samples were considered as L1F/L1R false positive cases. There were no L1F/L1R false negative samples and there was an excellent agreement of

96.2% (78 of 81 samples) between the two systems (p=2,58*10-19, two-tailed Fisher Exact Probability Test).
L1F/L1R's estimated sensitivity was 91,1% (31/34) and estimated specificity was 100% (47/47). The discordant results are based on the higher sensitivity attributable to the PCR technique used by the L1F/L1R test and also on the already known cross-reactions of HCII (HPV 6, 42, 53). We have findings showing HCII cross-reactions with other genotypes (HPV 30, 82) as well, but this was not further investigated.
After additional tests (semiquantitative determination of the amount of HPV DNA, individual genotyping, and also additional analytical data) the 3 false positive samples may be classified differently. We tried to detect a cross-reaction between HPV 90 and HPV 45 (HPV 90 was proved to be present by sequencing, where HPV 45 was detected by the L1F/L1R system), but we were not able to detect any cross-reaction using pure DNA. As a conclusion both HPV 90 and HPV 45 DNA were probably present in the sample and sequencing could not provide information about the minor components of the mixed infections.
In order to further test the L1F/L1R system, samples issued as ASCUS from a cohort of 360 persons were tested. There were 198 negative samples, while 162 were positive, of which 120 samples showed to contain only one HPV type, 33 cases showed infection with 2 HPV types, and in 9 cases multiple infections with 3 HPV types was found. The most frequent type was HPV 16, which was encountered in 36 cases, followed by HPV type 31 in 23 cases. 19 cases presented infections with low-risk HPV types. We compared our results with data obtained by Coutlee et al. (Coutlee et al., 2002)by superimposing the percentages from prevalence of 14 high-risk HPV types, and we found similar distributions, slightly influenced probably by the populational differences. Statistical analysis shows strong correlation between the results of the two systems. Both the Wilcoxon signed-ranks test ($t= 1.005398$, $P< 0.05$) and the Mann-Whitney U-test ($z= -0.781111$, $P< 0.05$) show high concordance between the two systems.

In the cervical samples of our 2,215 patients with PID (Group 1) the most prevalent agent was HPV as it was detected in 33.74%. In patients without signs or symptoms of PID (Group 2), similarly to group HPV was the most frequently detected agent, its frequency (26.40%) was significantly lower than in Group 1 ($p<0.001$).

With aid of experimental design method, namely Taguchi optimization we were able find optimum solution for different multifactorial optimization problems, for the enzyme linked immunosorbent assay (ELISA) and other biomolecular interactions e.g. DNA hybridisation with a versatility making it easy to tailor the method to a particular application and in this thesis also a highly multiplex PCR was optimized using method, very effectively.
Diagnosis of HPV infection requires the specific and sensitive detection of viral DNA. The L1 region of the HPV genome has been used for the development of widely applied and general PCR primer sets, such as MY09/11, PGMY, SPF and GP5+/6+ (Kleter et al., 1999). In this thesis we described a novel human papillomavirus detection method, which shows significant improvements over the previously published methods. To achieve sensitive, not biased amplification of the genotypes, a multiprimer consensus primer system was devised.
Recent advances in HPV vaccination (Munoz et al., 2004) (Berencsi et al., 2006) require the exact genotyping favorably based on those regions of the viral nucleic acid, which are evolutionary responsible for genotype-specific segregations.
The parallel use of genus- and genotype specific detections improves overall reliability of the detection. Moreover the genus specific approach itself allows the detection of new, unknown HPV types, and provides semi-quantitative information on the HPV DNA amount. In our practice we have found concordant genus and genotype specific signals in the cases of single infections, which is possibly a tool to rule out multiple infections.
The achievable performance was evaluated using a modified Taguchi optimization method. According to the results there is a slightly

better solution than the benchmark (L1F/L1R) setup. As it is proved in other systems (at low multiplicity) that the primer concentration is usually has a concentration optimum (Linz et al., 1990). The benchmark system was developed in a stepwise manner and its performance is excellent providing sensitivity below 100 copies per reaction for more than twenty genotypes. The development of these systems took a considerably long developmental time, however the Taguchi optimization achieved a slightly better solution in only one experiment proving its extraordinary efficacy. We used this experiment to evaluate our previous benchmark reaction, if we would have found a more advantageous setup.

We compared the L1F/L1R system to Hybrid Capture II (HCII) to assess clinical performances. HCII has a high reputation in this field, and the wide clinical application of this test makes it a standard in many aspects. Taking together the original HCII and the sequencing results the analysis revealed excellent performance of the L1F/L1R, 78 of 81 samples being concordant.

We determined the prevalence of the genotypes on a cohort of 360 persons. A very similar distribution of genotypes was published by Coutlee at al. (Coutlee et al., 2002)using the PGMY system, statistical analysis indicating close concordance between the two systems. In patients with PID the prevalence of cervical HPV infection was found to be significantly higher than a patient without PID.

The clinical application of these diagnostic procedures is constantly expanding, national authorities consider the introduction or already introduced the HPV testing in screening programs to improve the effectiveness of such preventive measures. Epidemiologic studies report that countries where HPV screening has been implemented, the incidence of cervical cancer has shown decreasing tendencies (Berkhof et al., 2005). Vaccination soon would further increase the demand for this testing. With this background the improvement of the current diagnostic procedures is mandatory to eliminate the still existing problems.

CONCLUSIONS

A new experimental design method was devised in the development of new human diagnostic devices. To demonstrate the application of Taguchi optimization, the examples of an ELISA and a PCR method were used. The method is fast, accessing the results in one step, compared to the traditional, time-consuming 'one-step-at-a-time' method. We exemplify the procedure with methods to optimize the detection of ScFv single chain .fragment of variable phages by ELISA and a PCR system. The efficacy of the Taguchi method is further improved by a special assignment of factors, which makes possible to calculate the biochemical parameters of the ELISA procedure carried out under optimum conditions. The optimum primerset concentration in the case L1F/L1R PCR system, which is a highly multiplex PCR method detecting human papillomavirus is proved, with an improved Taguchi optimization.

A new PCR based detection system was developed which detects a broader spectrum of HPV genotypes, is dynamic to recognize newly emerging HPV types, yields a balanced amplification and features an automatizable genotyping method (hybridization). The novel approach using two separate regions for the genus- and genotype specific detection of the amplicons allows highly optimized probe design for both applications. We created a new PCR detection system, using an optimized primer set for amplification and an automatizable genotyping approach.

We compared it to other systems and also tested its analytical and clinical performances. We compared the L1F/L1R system to Hybrid Capture II (HCII) to assess clinical performances. HCII has a high reputation in this field, and the wide clinical application of this test makes it a standard in many aspects. This clinical evaluation study revealed excellent performance of the L1F/L1R for the examined parameters. L1F/L1R's estimated sensitivity was 91,1% and estimated specificity was 100%. Taking together the original HCII and the verifying sequencing results the analysis revealed excellent performance of the L1F/L1R, 78 of 81 samples being concordant.

As a part of the clinical evaluation the pelvic inflammatory disease studied as a predictor of cervical cancer. HPV prevalence was 33.74% in patients with PID and 26.40% in the group of women without PID ($p < 0.001$). This suggests that patients suffering from PID apparently have a higher risk for aquiration of HPV infections.

Publications

Publications directly related to the thesis

Jeney C, Takacs T, Sebe A, Schaff Z.
Detection and typing of 46 genital human papillomaviruses by the L1F/L1R primer
system based multiplex PCR and hybridization.
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