



Article

Implementation of Valid HPV Diagnostics for the Early Detection of Cervical Cancer in Molecular Pathology: HPV 3.5 LCD-Array (Chipron GmbH) vs. PapilloCheck® (Greiner Bio-One GmbH) vs. VisionArray® (ZytoVision GmbH)

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Abstract: The occurrence of cervical cancer is often linked to a previous infection with a human papillomavirus (HPV). In order to detect HPV infections in cervical smears, a broad range of tests can be used. This study compares the two hybridisation-based DNA-microarray systems “HPV 3.5 LCD-Array” (Chipron GmbH) and “PapilloCheck®” (Greiner Bio-One GmbH), based on their ability to detect and differentiate HPV infections in 42 different cervical smears. PapilloCheck® can detect and individually identify 24 HPV types, whereas the 3.5 LCD-Array can differentiate among 32 HPV genotypes. However, both systems include all 13 high-risk (HR)-classified types. With Chipron having already stopped the production of the 3.5 LCD-Array test, quite a few laboratories are confronted with the need to establish a new HPV testing method. The two methods were found to have a high agreement regarding the clinical significance of the detected HR HPV types. Discrepant cases were additionally validated with the help of a third test (VisionArray® HPV, ZytoVision GmbH). The results of the VisionArray® test corresponded rather well with the results of the 3.5 LCD-Array.

Keywords: cervical cancer; cervical smear; subtyping; HPV; infection; hybridisation; array



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1. Introduction

Cervical cancer is the fourth most common and deadly cancer in women worldwide. The mean age at diagnosis in Germany is 53 years [1]. An infection with a human papillomavirus (HPV) can cause diseases ranging from ordinary warts to cervical cancer [2]. In almost all cases of cervical cancer, an association with an HPV infection can be established [3]. Approximately 10% of transient infections develop into persistent infections, which may develop into precancerosis, also known as cervical intraepithelial dysplasia (CIN) [4–6]. Without screening measures and therapies, this stage evolves into cervical cancer within five to ten years [7–9].

A human papillomavirus is a circular, non-enveloped, double-stranded DNA virus that has a genome size of approximately 8 kb, encoding eight genes: the early genes E1, E2,

and E4–E7, and the late genes L1 and L2, containing sequences that control viral replication and transcription. They are controlled by the large control region (LCR) and are activated during different phases of the HPV life cycle [10–14].

HPV genotypes can be further divided into three groups according to their carcinogenic potential. According to the International Agency for Research on Cancer (IARC) of the World Health Organisation (WHO), the HR group contains twelve HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), the intermediate-risk (IR) group seven (HPV 26, 53, 66, 67, 70, 73, and 82) and the low-risk (LR) group includes the rest. Type 68 is classified as likely carcinogenic, and for reasons of simplification, is also included in this group. Intermediate types are those with possible carcinogenicity, being more often found in squamous intraepithelial lesions than in cancers [15,16]. LR HPV types are mainly known to induce genital warts, of which 90% are caused by HPV types 6 and 11 [17]. Several open reading frames, as well as alternative splicing, lead to the different HPV types. More than 50% of all HPV-induced cervical cancers are caused by HPV type 16. An additional 15% are caused by HPV type 18 [18–25].

Like most viruses, HPV depends on the replication machinery of the host cell. To infect a cell, HPVs bind to extracellular heparin sulphate proteoglycans in lesions in the *Stratum basale*, leading to conformational changes in the L1 and L2 capsid proteins and a subsequent macropinocytosis-like process. After entering a cell, the virus loses its envelope and is integrated into the host cell's genome. In a next step, viral E1 and E2 proteins recruit the DNA polymerase and other essential cell-own enzymes for replication. Meanwhile, the proteins E6 and E7 increase cell proliferation to enable the replication of the viral genome during the S-phase (*Stratum spinosum*). This infection can either remain unproductive of new virions or become active. As soon as a cell is differentiated completely and reaches the surface (*Stratum granulosum*), the genes L1 and L2 are activated, at which time the capsid is formed, and the virus is put together. As soon as an infected cell detaches from the surrounding tissue, the new virions are released [26–32].

HPV testing as a diagnostic tool in routine screenings has led to the development of plenty of tests. According to Poljak et al. (2015 [33]), 193 tests are on the market, including DNA, RNA, and protein tests. For this study, two HPV hybridisation tests (PapilloCheck® (Greiner Bio-One GmbH), CE-IVD certified, and the routinely used, well-established and validated by a Germany-wide ring trial, but not CE-IVD certified, HPV 3.5 LCD-Array (Chipron GmbH)) were directly compared with each other regarding results, hands-on times, costs, sensitivity, and specificity. Discrepant cases were further analysed with the help of a third test (VisionArray® (ZytoVision GmbH, CE-IVD certified)). Furthermore, this study aimed at establishing a reliable detection process of HPV for routine diagnostics in molecular pathology in order to implement the 2017-passed In Vitro Diagnostic Medical Devices Regulation (IVDR), which has to be applied by 2025 at the latest. From then on, only CE-IVD-certified medical devices and procedures will be authorised for the use in routine diagnostics. Further requirements for the use of HPV tests and preventive measures are defined in the S3 guideline “Prävention des Zervixkarzinoms” (AWMF-Nr. 015/027OL), published by the “Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften e.V.” (AWMF), “Deutsche Krebsgesellschaft e.V.” (DKG) and “Deutsche Krebshilfe” (DKH) [34]. At the time of submission (summer 2024), Chipron has already stopped the production of the 3.5 LCD-Array test. Therefore, several routine laboratories are forced to implement a new HPV testing method as soon as possible.

2. Materials and Methods

2.1. Collective of Patients

Overall, 42 cervical smears of women aged 37 to 75 years (mean: 50 years, median: 48 years) were randomly selected according to sufficient amounts of tissue and DNA concentration and analysed. The samples were provided by cooperating gynaecologists and collected in the period from August 2020 until January 2021 for clinical routine diagnostic purposes. Among them, 35/42 (83.3%) had no HPV vaccination, while the rest did not specify.

2.2. Sample Preparation

The DNA was extracted using the test-specific oCheck[®] DNA Extraction Kit (Greiner Bio-One International GmbH, Kremsmünster, Austria) [35] and the QIAamp[®] DNA Micro Kit (50) (Qiagen N.V., Venlo, The Netherlands) [36], according to the manufacturer's instructions.

2.3. HPV Genotyping

After the DNA extraction, the HPV types were identified using the HPV 3.5 LCD-Array test (Chipron GmbH, Berlin, Germany) [37] and the PapilloCheck[®] test (Greiner Bio-One International GmbH, Kremsmünster, Austria) [38], according to their respective protocols. Different extraction kits were used according to the manufacturer's instructions (cf. also Section 2.2). Discrepant cases, extracted with both extraction methods, were additionally validated with the help of the VisionArray[®] HPV Test (ZytoVision GmbH, Bremerhaven, Germany).

HPV types that could not be detected by all three kits were excluded (grey, see Table 1) in order to enable comparative analyses, leaving 22 different genotypes (white) in 42 samples. To further simplify the results, HPV 68 was classified as an HR HPV type, as already done in other studies before.

Table 1. Overview of the different HPV genotypes that each of the used tests is able to detect according to their respective design. A dark grey field shows which HPV type cannot be detected by one or more of the used tests. Each HPV type marked white was therefore excluded for comparison reasons.

HPV Type	3.5 LCD-Array	Papillo-Check [®]	VisionArray [®]	HPV Type	3.5 LCD-Array	Papillo-Check [®]	VisionArray [®]
16 (HR)				70 (IR)			
18 (HR)				73 (IR)			
31 (HR)				82 (IR)			
33 (HR)				6 (LR)			
35 (HR)				11 (LR)			
39 (HR)				40 (LR)			
45 (HR)				42 (LR)			
51 (HR)				43 (LR)			
52 (HR)				44 (LR)			
56 (HR)				54 (LR)			
58 (HR)				55 (LR)			
59 (HR)				57 (LR)			
68 (HR)				61 (LR)			
26 (IR)				62 (LR)			
34 (IR)				72 (LR)			
53 (IR)				81 (LR)			
66 (IR)				83 (LR)			
67 (IR)				84 (LR)			
68a (IR)				90 (LR)			
68b (IR)				91 (LR)			
69 (IR)							

2.4. Agreement Analysis

Analytical sensitivity indicates the rate of true positive cases, described as the ratio of correctly determined positive results to the total number of positive results:

$$\text{sensitivity} = \frac{\text{number of true positive cases}}{\text{number of true positive cases} + \text{number of false negative cases}} \quad (1)$$

Analytical specificity is a measure of true negative cases, or the ratio of correctly determined negative results to the total number of negative results:

$$\text{specificity} = \frac{\text{number of true negative cases}}{\text{number of true negative cases} + \text{number of false positive cases}} \quad (2)$$

With the help of Cohen's kappa (κ) the agreement between two tests can be determined:

$$\kappa = \frac{\text{relative agreement} - \text{hypothetical probability of agreement}}{1 - \text{hypothetical probability of agreement}} \quad (3)$$

2.5. Ethics

Institutional Review Board Statement: Tissue/tumour samples and/or patient data used in this study were provided by the University Cancer Center Frankfurt (UCT). Patient consent was waived due to the approval by the institutional Review Boards of the UCT and the Ethical Committee at the University Hospital Frankfurt (project-number: SGO-1-2024).

Informed Consent Statement: Patient consent was waived due to the approval by the institutional Review Boards of the UCT and the Ethical Committee at the University Hospital Frankfurt (project-number: SGO-1-2024).

3. Results

3.1. 3.5 LCD-Array Versus PapilloCheck®

As a first step, the whole collective of 42 samples was collectively tested with both the 3.5 LCD-Array and PapilloCheck®. According to the HPV 3.5 LCD-Array test 12 out of 42 samples (28.6%) were tested positive (HPV+) for HPV: all of them had no HPV vaccination (100%). After excluding cases with detected subtypes that could only be detected with the LCD-Array, 10 samples remained (10/42, 23.8%, Table 2). 19 out of the 42 (45.2%) samples tested with PapilloCheck® showed an infection with HPV, 18 of which had no vaccination (94.7%) (cf. Table 2).

Table 2. Number of detected HPV Types and infections per test.

HPV-Detection & Subtypes (n = 42)	3.5 LCD-Array	PapilloCheck®
HPV–	32 (76.2%)	23 (54.8%)
HPV+	10 (23.8%)	19 (45.2%)
Single HPV+	7 (70%)	12 (63.2%)
Multiple HPV+	3 (30%)	7 (36.8%)
HR HPV	7 (50%)	12 (40%)
IR HPV	6 (42.9%)	17 (56.7%)
LR HPV	1 (7.1%)	1 (3.3%)

3.1.1. The HPV 3.5 LCD-Array Kit

A total of 6 HR HPV types (16, 31, 33, 51, 52, 58), 2 IR HPV types (53, 66), and 1 LR HPV type (42) was detected. A total of 14 HPV subtypes was detected in 10 positive single or multiple infections, including 7 HR, 6 IR, and 1 LR HPV types. In 7 out of these 10 positive

cases, only a single HPV infection was detected. The other three samples showed two or three different HPV types. The highest number of coinfections was three and was detected in only one case.

3.1.2. PapilloCheck®

Here, 8 HR HPV types (16, 31, 33, 39, 51, 52, 58, 68), 3 IR HPV types (53, 66, 70) and 1 LR HPV type (42) were found. Added together, 30 HPV subtypes were detected in 19 positive cases as single or multiple infections, containing 12 HR, 17 IR, and 1 LR HPV type. Twelve of these 19 positive cases were tested positive for only one HPV type. Multiple infections were detected in 7 samples. The highest number of coinfections detected in one sample was 5.

The HPV subtype that was detected most in both assays and as single (2×) or multiple (3×) infection was IR HPV type 53 (9 times in PapilloCheck® vs. 4 times in the 3.5 LCD-Array). The most detected HR HPV type was 31 (2 single, 3 multiple infections). HPV 16 was detected once in the 3.5 LCD-Array and twice in PapilloCheck®. HPV 39 and 68 were not detected by the 3.5 LCD-Array. With the exception of HPV types 33, 51, 52, 58, and 42, for which no difference could be observed for the number of detections, all other types were detected more frequently by PapilloCheck®. HPV 68 and 70 were not detected by the 3.5 LCD-Array. Critical HR type HPV 18 was not detected at all in the whole sample collective.

Table 3 shows that among all the single infections detected by PapilloCheck®, 41.7% contained an infection with a HR HPV type, whereas the single infections detected by the 3.5 LCD-Array contained an infection with a HR HPV type in 57.1% of all cases. Of the 3 multiple infections detected by the 3.5 LCD-Array, 2 showed 2 infections and 1 showed 3 infections. Five of the 7 samples with multiple infections detected with PapilloCheck® were double infections, 1 contained 3 different HPV types and the last one 5 different HPV types. The two samples with at least 3 coinfections contained 1 or more HR HPV type each, as successfully detected by PapilloCheck®. The 3.5 LCD-Array could not detect the HR type in the sample with 5 coinfections. From 2 samples with 2 coinfections, each the 3.5 LCD-Array did not detect a single HR type. PapilloCheck® could detect a HR infection in 2 out of 5 samples.

Table 3. Number of detected HPV types and coinfections and their respective share of HR types.

Number of Detected HPV Types/Sample (x-Fold Infection)	Number of Samples n = 42		Number of Positive HR HPV Samples	
	3.5 LCD-Array	PapilloCheck®	3.5 LCD-Array (n = 10 HPV+)	PapilloCheck® (n = 19 HPV+)
0 (HPV−)	32 (76.2%)	23 (54.8%)	0 (0%)	0 (0%)
1 (single)	7 (16.7%)	12 (28.6%)	4/7 (57.1%)	5/12 (41.7%)
2 (double)	2 (4.8%)	5 (11.9%)	0/2 (0%)	2/5 (40%)
3 (triple)	1 (2.4%)	1 (2.4%)	1/1 (100%)	1/1 (100%)
5 (fivefold)	0 (0%)	1 (2.4%)	0 (0%)	1/1 (100%)

A direct comparison of the results (only positive or negative) between the 2 assays showed discrepancies in 9/42 samples (21.4%).

Together, both systems detected 12 HPV types: 8 HR, 3 IR, and 1 LR HPV type (cf. Tables 3 and 4). The detection of HPV 33, HPV 51, HPV 52, HPV 48, and HPV 42 had a concordance of 100% between the two detection systems.

Table 4. Number of detected HPV types by 3.5 LCD-Array and PapilloCheck® and their concordance.

Risk Group	HPV Type	Number of Detections with 3.5 LCD-Array	Number of Detections with PapilloCheck®	Concordance
HR	HPV16	1	2	1 (50%)
	HPV 31	2	3	2 (66.7%)
	HPV 33	1	1	1 (100%)
	HPV 39	0	2	0 (0%)
	HPV 51	1	1	1 (100%)
	HPV 52	1	1	1 (100%)
	HPV 58	1	1	1 (100%)
	HPV 68	0	1	0 (0%)
IR	HPV 53	4	9	4 (44.4%)
	HPV 66	2	7	2 (28.6%)
	HPV 70	0	1	0 (0%)
LR	HPV 42	1	1	1 (100%)

3.2. Discrepant Samples in Detail

13/42 (31.0%) samples showed a discrepant result also regarding HPV types (cf. Table 4). In 9 of them, the 3.5 LCD-Array could not detect an HPV infection, whereas PapilloCheck® could detect at least one type. The other 4 showed a different result regarding the respective HPV type. PapilloCheck® could detect more multiple infections. In 4 samples (1, 13, 19, and 21), PapilloCheck® could detect an infection with a HR HPV type (HPV 16, 39, or 68) that the 3.5 LCD-Array could not. HPV 16 was detected once by the 3.5 LCD-Array and twice by PapilloCheck®. IR HPV types 53 and 66 were detected significantly more often by PapilloCheck®.

To further evaluate discrepant results, SNR-values (signal-to-noise-ratio) were assigned for each HPV type detected by PapilloCheck® (cf. Table 5). In case numbers 1, 10, 13, 16, and 33, SNR-values < 100 were measured. These values are close to the detection limit. The lowest SNR-value measured was 25.4 for HPV 16 in sample 1. The other 4 samples with SNR-values < 100 were associated with HPV 53. Except for HPV 33 in sample 10, all concordant detections had a SNR-value > 1000. Only 2 discrepant detections had a SNR-value > 1000 (HPV 53 in sample 15 and HPV 31 in sample 33). HPV 53 in sample 15 was, with 3485.7, also the highest detected SNR-value of all.

Table 5. SNR-Values of discrepant samples after analysis with the 3.5 LCD-Array and PapilloCheck®.

Case No.	3.5 LCD-Array	PapilloCheck®	
	Result	HPV Type	SNR-Value
1	HPV-	16 (HR)	25.4
7	HPV-	66 (IR)	229.3
10	16 (HR) 31 (HR) 33 (HR)	16 (HR)	1958.7
		31 (HR)	1134.7
		33 (HR)	879.2
		53 (IR)	33.0
		66 (IR)	608.9
12	HPV-	66 (IR)	539.5
13	HPV-	39 (HR)	160.9
		53 (IR)	37.9
15	HPV-	53 (IR)	3485.7
16	HPV-	53 (IR)	79.3
17	HPV-	66 (IR)	211.0

Table 5. Cont.

Case No.	3.5 LCD-Array	PapilloCheck®	
	Result	HPV Type	SNR-Value
19	53 (IR)	53 (IR) 68 (HR)	2538.2 530.6
21	HPV–	39 (HR)	310.2
25	HPV–	66 (IR)	134.2
33	42 (LR)	31 (HR) 42 (LR) 53 (IR)	1255.7 1927.8 29.6
37	51 (HR)	51 (HR) 70 (IR)	1728.4 110.3

3.3. Quality Parameters: Sample Concentrations and Fragment Lengths

The lowest measured total concentration after DNA extraction was 1.28 ng/μL (sample number 25), the highest 167.00 ng/μL (sample number 37). Both concentrations were observed with the use of the QIAamp kit. With oCheck®, the extracted samples had concentrations ranging from 4.00 ng/μL (sample 21) to 114.00 ng/μL (sample 37). Five samples extracted by each kit had DNA concentrations < 10 ng/μL (QIAamp: Samples 12, 13, 16, 19, and 25; oCheck®: Samples 1, 12, 16, 19, and 21), all of which belonged to the group of discrepant cases. Therefore, samples 12, 16 and 19 had low DNA concentrations after both extraction methods.

The amplifiable fragment lengths of the DNA extracted with both kits ranged between 200 bp and 600 bp and were therefore comparable. However, only once was the fragment length 200 bp, indicating insufficient quality (case number 33, extracted with the QIAamp kit, belonging to the discrepant cases group). Twice the fragment length was 300 bp (case numbers 15 and 21, also extracted with QIAamp and also yielding discrepant results). Most DNA's extracted with QIAamp had a fragment length of 400 bp (9/13, 69.2%), whereas most DNA's extracted with the oCheck® had fragment lengths of 600 bp (10/13, 76.9%). In all cases, fragments extracted with oCheck® were as long as the ones extracted with QIAamp or even longer.

3.4. Agreement Analysis

The sensitivity of PapilloCheck® came to 100% and the specificity to 71.88%. The calculation was done using the 3.5 LCD-Array as a reference and according to the criteria of the “Deutsche Akkreditierungsstelle” (DakkS) [39].

With a Cohen's kappa (agreement) coefficient κ of 0.55, the result conformity between the 3.5 LCD-Array and PapilloCheck® was moderate. Substantial to perfect agreement ($\kappa = 0.61$ –1) was registered for HR HPV types 16, 31, 33, 51, 52, and 58 and LR type HPV 42. No agreement was detected for HR HPV types 39 and 68 or IR HPV type 70.

3.5. LCD-Array/PapilloCheck® Versus VisionArray® HPV to Verify Discrepant Cases

Due to discrepant results in 13 of 42 cases, these cases were analysed again, this time using the VisionArray® HPV Assay (ZytoVision, cf. Table 6). The results were compared excluding HPV types that could not be detected by all three tests. Two cases could not be integrated into this comparative analysis, since they did not have enough material left (samples 10 and 15). Seven previously with QIAamp extracted samples (7/11, 63.6%) were HPV-negative. Four samples (4/11, 36.4%) were HPV positive, 2 of them as single and 2 as multiple infections. In comparison to this, the 3.5 LCD-Array detected 8/11 cases (72.8%) as negative and 3/11 (27.2%) as positive. All 3 positive cases were single infections. Seven out of 11 of the samples extracted with oCheck® were negative and

4 positive, 2 of them as single and 2 as multiple infections, confirming the results of the QIAamp/VisionArray[®] analysis.

Table 6. Number of cases with HPV infections and types detected by the 3.5 LCD-Array, PapilloCheck[®], and VisionArray[®] and their respective extraction method in the 11 discrepant cases.

HPV Detection & HPV Types	3.5 LCD-Array (QIAamp)	PapilloCheck [®] (oCheck [®])	VisionArray [®]	
			QIAamp	oCheck [®]
HPV–	8	0	7	7
HPV+	3	11	4	4
Single HPV+	3	7	2	2
Multiple HPV+	0	4	2	2
HPV (HR)	1	5	3	3
HPV (IR)	1	10	2	3
HPV (LR)	1	1	1	2

Overall, the agreement of the different HPV genotypes among all of the used extraction/detection methods was 100% for HR HPV types, 66.7% for LR, and 50% for LR HPV types.

HPV types 51, 53, and 42 were the only ones detected by all extraction/detection methods (cf. Figure 1). The combination of oCheck[®] and PapilloCheck[®] was the only one detecting the HR HPV types 16 and 39 and the possibly cancerogenic HPV type 70. oCheck[®] and VisionArray[®] was the only pairing that could detect HPV 6.

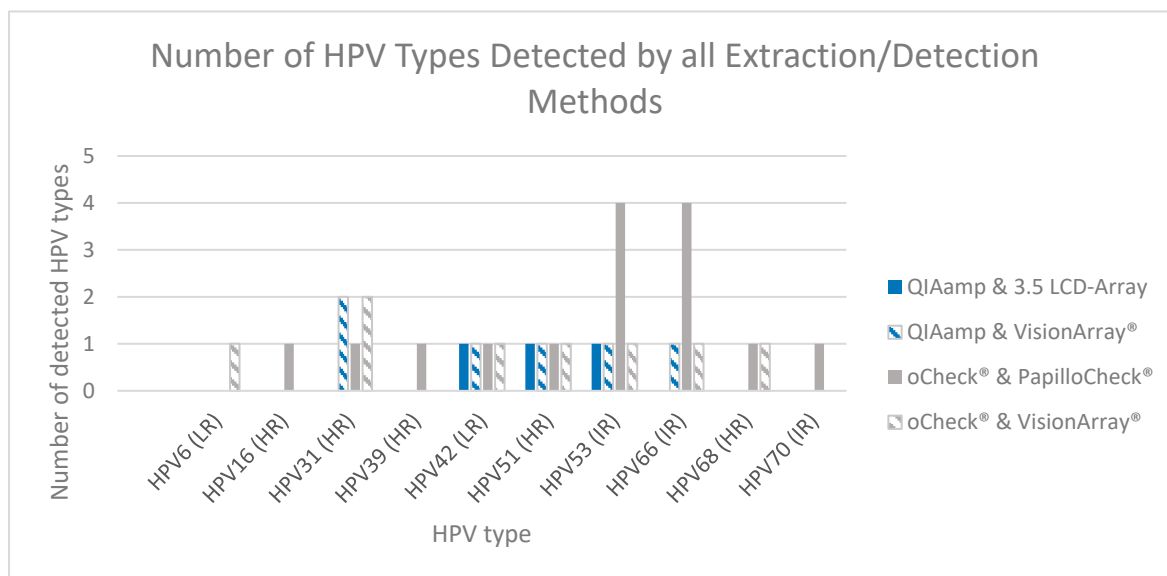


Figure 1. Number of HPV types detected by the four different extraction/detection methods. HPV types 51, 53, and 42 were the only ones detected by the 3.5 LCD-Array and simultaneously by all extraction/detection methods. The combination of oCheck[®] and PapilloCheck[®] was the only one detecting the HR HPV types 16 and 39 and the possibly cancerogenic HPV type 70. oCheck[®] and VisionArray[®] was the only pairing that could detect HPV 6.

Table 7 shows the HPV types detected by the 3.5 LCD-Array, PapilloCheck[®], and VisionArray[®] for each sample. Discrepancies are marked bold. The results obtained by the combination of oCheck[®] and PapilloCheck[®] deviated in each of the 11 samples from the results of the other 3 extraction/analysis methods regarding HPV detection and/or HPV type. Seven samples (1, 7, 12, 13, 16, 17, 21, 7/11, 63.6%) were negative in both QIAamp/3.5 LCD-Array and QIAamp/VisionArray[®]. The 3.5 LCD-Array was the only

test that did not detect a multiple infection within this cohort of discrepant cases. The highest concordance, with 72.7% (8/11 samples, 7 of them negative), was achieved between the QIAamp extracted and with either 3.5 LCD-Array or ZytoVision analysed samples. Cohen's kappa was here for the solely detection of an HPV infection 0.79 and showed for the specific HPV type a $\kappa = 0.6$. 3/4 (75.0%) of the in the 3.5 LCD-Array, as positive detected samples were either confirmed by ZytoVision, or even more HPV types were detected there. Substantial to perfect agreement ($\kappa = 0.61$ –1) was achieved for HR HPV type 51, IR type 53, and LR type 42. No agreement was registered for HPV types 31 (HR) or 66 (IR).

Table 7. Comparison of HPV types after extraction with QIAamp or oCheck[®] and detection by the 3.5 LCD-Array, PapilloCheck[®] and VisionArray[®].

Case No.	QIAamp [®]		oCheck [®]	
	3.5 LCD-Array	VisionArray [®]	VisionArray [®]	PapilloCheck [®]
1	HPV–	HPV–	HPV–	16 (HR)
7	HPV–	HPV–	HPV–	66 (IR)
10	16 (HR) 31 (HR) 33 (HR)	no material left for a validation		16 (HR) 31 (HR) 33 (HR) 53 (IR) 66 (IR)
12	HPV–	HPV–	66 (IR)	66 (IR)
13	HPV–	HPV–	HPV–	39 (HR) 53 (IR)
15	HPV–	no material left for a validation		53 (IR)
16	HPV–	HPV–	HPV–	53 (IR)
17	HPV–	HPV–	HPV–	66 (IR)
19	53 (IR)	53 (IR) 31 (HR)	6 (LR) 31 (HR) 53 (IR) 68 (HR)	53 (IR) 68 (HR)
21	HPV–	HPV–	HPV–	39 (HR)
25	HPV–	66 (IR)	HPV–	66 (IR)
33	42 (LR)	31 (HR) 42 (LR)	31 (HR) 42 (LR)	31 (HR) 42 (LR) 53 (IR)
37	51 (HR)	51 (HR)	51 (HR)	51 (HR) 70 (IR)

Cohen's kappa for oCheck[®]/PapilloCheck[®] or VisionArray[®] was 0 for the detection of HPV (none to slight agreement) and 0.4 (moderate agreement) for the subtyping. The highest concordance here was detected for HPV types 42, 51, and 68, followed by HPV type 31. None to slight agreement was reached for HPV types 6, 16, 39, and 70.

4. Discussion

Being most commonly sexually transmittable, not only men, but also most women have at least one HPV infection in their life which is often linked to developing cervical cancer, making it highly important for laboratories to establish a valid and reliable method for the detection of an HPV infection in routine diagnostics. Since the guidelines passed by the “Gemeinsamer Bundesausschuss” (G-BA), women aged 20–34 have the right to have an annual genital screening. Therefore, two HPV tests (3.5 LCD-Array and PapilloCheck[®]) were compared with each other regarding results, hands-on-times and costs, sensitivity, and specificity. Discrepant cases were further analysed using a third HPV test (VisionArray[®]). Currently, there is no study comparing the results of the 2 or 3 tests, respectively. Through

the individual test designs, the HPV 3.5 LCD-Array Kit (Chipron) is able to detect and differentiate among 32 HPV genotypes, including all 13 HR types, 6 IR types, and 13 LR types. The primer pairs used cover highly conserved motifs within the HPV L1 gene. The PapilloCheck® (Greiner Bio-One) is able to detect and individually identify 24 HPV types. It contains all 13 HR types, as well as 5 IR types and 7 LR types. A highly conserved motif within the E1 gene of the viral DNA is covered here. A third test was consulted for cases that were discrepant after analysis with either of the two tests. The VisionArray® includes all 13 HR types, 11 IR types, and 17 LR types and amplifies the L1 region of the HPV genome as well. In order to be able to compare the results, types that could not be detected by all three tests were excluded from this study, leaving 22 different genotypes, with 13 of them being HR HPV types. Nine of the 42 (21.4%) samples analysed with the 3.5 LCD-Array and PapilloCheck® showed discrepant results and were further analysed with the VisionArray®.

All HR HPV types could, in theory, be detected with all three HPV test kits. Additionally, 5 IR (HPV 53, 66, 70, 73 and 82) and 3 LR (HPV 6, 11, 42) HPV types could be detected by all of them. Together these HPV tests cover a broad range of HPV types. Comparing the hybridisation results, the 3.5 LCD-Array with PapilloCheck® yielded a higher concordance for the subtyping than for the detection (HPV yes or no) in general. All three assays were able to detect multiple infections simultaneously. Most samples with multiple infections were detected by PapilloCheck® (36.9%), the least from the 3.5 LCD-Array (30.0%), with up to three infections at once for the 3.5 LCD-Array and up to 5 for PapilloCheck®. Many smears with multiple infections showed at least one HR HPV type (3.5 LCD-Array: 33.3%, PapilloCheck®: 57.1%, VisionArray®: 100.0%), correlating with studies from Senapati et al. (2017) [40] and Herrero et al. (2005) [41]. They concluded that multiple infections often indicate an HR type infection, making it extremely important for HPV tests to detect all infections reliably. Special attention should be paid to HR HPV types, since they have the highest clinical relevance. Often with cervical cancer, associated HR HPV types 16, 31, 33 and 58 were detected with a high agreement ($\kappa = 0.86$). Most relevant HR HPV type 16 was detected once with the 3.5 LCD-Array and twice with PapilloCheck®. In the subsequent analysis with the VisionArray®, HPV 16 could not be detected in the discrepant sample, just like with the 3.5 LCD-Array. Unfortunately, previous screening data were insufficiently collected for the whole sample collective, which would have been especially important for this particular HPV 16-positive case. Looking at the SNR-value from PapilloCheck® in this specific case showed a rather low value (25.4), corresponding also to the low DNA concentration. If possible, a repetition of the PCR and/or DNA extraction should be performed for this sample. IR and LR HPV types are important for the placement of the infection into the epidemiologic context. They are important for classifying benign lesions, for monitoring and prevention, for differential diagnoses, planning therapies, and for patient counselling. The detection of low-risk HPV types is therefore an important element for the individual patient care, as well as for public health strategies. There are European Medicines Agency (EMA)-approved and preventive vaccinations (Gardasil, Gardasil9 and Cervarix) that already include LR HPV types as well.

PapilloCheck® had a much higher detection rate, which could have several reasons. All three testing methods are completely genotyping tests, differing only in the target areas. Whereas the 3.5 LCD-Array and VisionArray® detect, as most of the available detection tests, the L1 gene of HPVs, PapilloCheck® detects the E1 gene, which could explain the higher concordance between the tests from Chipron and ZytoVision. Furthermore, different detection limits between PapilloCheck® and the 3.5 LCD-Array could be another reason for this discrepancy. A cut-off value of 20 copies/PCR as a valid amount for the detection of any HPV type was predefined by PapilloCheck®, while for HPV 16 this was set to 10 copies.

The 3.5 LCD-Array, however, specified its detection limit as 100 copies/PCR. These findings correlate with the SNR-values (signal-to-noise ratio) from PapilloCheck®. Eleven of the 13 discrepant cases showed SNR-values < 600, 4 at <40, while the concordant results showed SNR-values > 1000, correlating with more reliable results. The manufacturer indicates that a cut-off value < 20 classifies a sample as negative for that type, which also underlines these findings. Low SNR-values (<100) seem to indicate low initial DNA amounts of the respective HPV type or a false positive result. According to Carneiro et al. (2013 [39]), these cases may be initial infections or spontaneous remissions. Higher SNR values might have a higher chance of recurrence or persistent infection. In general, each HPV type has its own specific detection limit and has therefore, in cases of discrepancies, to be evaluated individually. The overall quality of the DNA extraction with oCheck® seems to be slightly better. However, only PapilloCheck® relies on its own specific extraction method. The influence of the extraction method can be evaluated by the third independent analysis method. Different primers and their designs could lead to different results as well. Contaminations could be excluded, as every sample showed a different result. Cut-off values that are set too low could lead to the overestimation of transient infections without pathological significance, leading to possible over-therapy and psychological consequences. On the other hand, cut-off values set too high could result in the missing detection of an infection, and therefore, no diagnostic confirmation and follow-up. The broad spectrum of detectable HPV types, also including IR and LR types, enables the coverage of multiple infections simultaneously and hints to the degree of dysplasia. However, the sensitivity should not be reduced as a consequence of higher amounts of detectable HPV types. Lower specificities could be attributed to the transience of HPV infections. However, since the age of women tested in this study was >37 years, the risk of temporary infections and therefore false positive results was reduced.

Even though overall validities of laboratory tests should have priority, temporal and financial aspects should not be neglected. Concerning the durations of the different extraction methods, no difference between the two could be observed. Both took about 60 min of hands-on-time. Major discrepancies became visible during incubation times. Whereas QIAamp requires 4–6 h of cell digestion/lysis and suggests even a digestion overnight, oCheck® takes only 45 min. The time between the procedures (PCR and detection) and the final report is approximately the same for all three tests. The costs for the two different extraction methods added up to approximately 5.50 € for each sample extracted with QIAamp and to more than 50% less (approx. 2.5 €/sample) for oCheck®. Prices for subsequent analyses were highest for the VisionArray®, followed by the 3.5 LCD-Array and PapilloCheck®. These prices are, of course, approximate values without guarantee and can vary depending on the individual company offer. Altogether, the combination of oCheck and PapilloCheck® was by far the fastest and most cost-effective method. Furthermore, PapilloCheck® could detect 12 samples at once, the 3.5 LCD-Array 8 and VisionArray® only 1 sample per chip, being proportional to the costs of each analysis.

In a next step, the patient collective should be enlarged to increase this study's validity. Furthermore, additional parameters should be included for the selection of patients prior to the testing, such as dysplasia or risk factors like the smoking status. Meijer et al. (2009 [38]) suggested a cohort of >500 samples with a diagnosed CIN2. Another possibility other than comparing results with gold standard tests to validate the results would be Next Generation Sequencing. Due to more and more implementations into routine diagnostics and the associated economic attractiveness, the already huge variety of HPV tests available on the free market is expected to further increase, making it even more important. In order to make a prognostic statement, all tested patients should be monitored further.

5. Conclusions

In conclusion, the manufacturers state that their products are only intended to be used as an addition to the differential diagnosis of cervical carcinoma and that therapeutic measures should not be initiated based on the test result alone, only in combination with previous findings and histopathological results. Further well-established diagnostic tools such as the PAP smear are still a good addition. All publications, including this one, however, agree regarding the urgency of standardising the detectable HPV types. Nonetheless, all three HPV tests cover a broad range of HPV types and yield valid results. Furthermore, all of them are easy to integrate into routine diagnostics of molecular pathology. Based on these results the VisionArray[®] was established for the use in routine diagnostics.

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