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Molecular PCA3 Diagnostics on Prostatic Fluid

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BACKGROUND. The PCA3 test on urine can improve specificity in prostate cancer (PCa) diagnosis and could prevent unnecessary prostate biopsies. In this study, we evaluated the PCA3 test on prostatic fluid and compared this with the PCA3 test on urine in a clinical research setting.
METHODS. Prostatic fluid and urine samples from 67 men were collected following digital rectal examination (DRE). The sediments were analyzed using the quantitative APTIMA® PCA3 test. The results were compared with prostate biopsy results.
RESULTS. Using a PCA3 score of 66 as a cut-off value, the test on prostatic fluid had 65% sensitivity for the detection of PCa, 82% specificity and a negative predictive value of 82%. At a cut-off value of 43, the test on urine had 61% sensitivity, 80% specificity and a negative predictive value of 80%.
CONCLUSIONS. The PCA3 test can be performed on both urine and prostatic fluid in the diagnosis of PCa with comparable results. *Prostate 67: 881–887, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer antigen 3; human; prostatic neoplasms; diagnosis; prostatic fluid; urine**

INTRODUCTION

Currently, prostate cancer (PCa) is the most common cancer in men in the United States of America. In 2006, PCa will be diagnosed in approximately 234,460 American men [1]. Since 27,350 American men will die of this disease, the ''incidence mortality ratio'' will be 8.57. This indicates that almost seven out of eight men with PCa will die *with it* and *not from it*.

For comparison, in Europe an estimated 225,227 men were newly diagnosed with PCa in 2002 and about 83,066 died from this disease (http://www-dep.iarc.fr/) [2]. This trend poses a dilemma for the urologist and biomarkers are urgently needed to aid in the treatment decision.

Serum prostate-specific antigen (PSA) is regarded as the standard diagnostic PCa marker. PCa awareness, leading to widespread use of PSA testing has led to a lower tumor stage and grade at the time of diagnosis. However, PSA is not cancer-specific, resulting in a high negative biopsy rate. Moreover, its use is associated with certain drawbacks, for example, the diagnosis of clinically irrelevant tumors (i.e., overdiagnosis) and potentially overtreatment [3].

As a result, there is an urgent need for PCa-specific markers that can improve the specificity in PCa diagnosis and can differentiate between indolent and aggressive disease. Therefore, new PCa markers have been identified and their diagnostic potential needs to

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be assessed in body fluids such as serum, plasma, urine, and prostatic fluid [3–13].

PCA3 is a prostate-specific non-coding RNA which is highly over expressed in more than 95% of primary PCa specimens and PCa metastases [14,15]. Hessels et al. [16] found that the median upregulation of PCA3 in PCa tissue compared with normal prostate tissue was 66-fold. Moreover, a median 11-fold upregulation was found in prostate tissues containing less than 10% of PCa cells. As PCA3 is a non-coding RNA, a dual time resolved fluorescence (TRF)-based RT-PCR assay was developed to explore the utility of PCA3 gene expression-based analysis to identify PCa cells in urinary sediments after digital rectal examination (DRE). Because the number of cells shed into the urine is likely to vary, we decided to use PSA as a normalization gene, since PSA mRNA expression is rather comparable for non-malignant and malignant prostate cells [17]. This TRF-based PCA3/PSA test yielded a negative predictive value of 90% in a population of men, admitted for prostate biopsies based on a serum PSA value >3 ng/ml. This first study showed that PCA3 has potential clinical diagnostic value and may provide for more accurate biopsy decisions [16]. Using the uPM3^{TM} test which is based on a quantitative nucleic acid sequence-based amplification (NASBA) technology also using PCA3 as a target, two independent studies confirmed these results [18,19]. Only last year, Groskopf et al. [20] introduced the quantitative automated probe transcription-mediated amplification (APTIMA®) PCA3 test which uses transcription-mediated amplification (TMA): a RNA transcription amplification system using RNA polymerase and reverse transcriptase to drive the isothermal reaction that allows the reaction to be carried out in a single tube format. Recently, Hessels et al. demonstrated that the results obtained with the APTIMA® PCA3 test using urinary sediments instead of whole urine are similar to those obtained with the TRF-based PCA3 test (Hessels et al. submitted). In conclusion, quantification of PCA3 gene expression using three established and validated technology platforms yields similar results, that is, an improved accuracy in the early detection of PCa.

The rationale for the molecular PCA3 test on urine is as follows. Both prostate and PCa cells shed into the prostatic ductal system. Upon DRE cells are mobilized towards the prostatic urethra and upon micturition they are flushed out with the first voided urine.

It is also known that occasionally during the DRE a patient will produce a few drops of prostatic fluid from the meatus. Naturally, this fluid should also contain prostate and PCa cells. Consequently, prostatic fluid could be a suitable substrate for the PCA3 test. Upon micturition the cells remaining in the (prostatic) urethra will be flushed out with the first voided urine.

Thus far, to our knowledge, nobody has used prostatic fluid samples for molecular diagnostic testing. In this study, we compared the diagnostic value of the APTIMA® PCA3 test on prostatic fluid with that on urine after DRE in 67 men who were to undergo prostate biopsies.

MATERIALS AND METHODS

Prostatic fluid and the first voided urine after DRE were collected from 67 men who were to undergo ultrasound-guided, transrectal, prostate biopsies as a result of an elevated serum PSA value or an abnormal DRE. In all men the DRE was performed by the same urologist (EBC) in the outpatient clinic of a community hospital (Hospital Group Twente (Region Hospital Midden-Twente), Hengelo, The Netherlands).

Previously all men had received study information and had signed their informed consent.

As part of standard clinical practice, both serum PSA and the fraction of free serum PSA had already been determined in most men.

All other samples and data were collected prospectively.

The DRE was performed according to a standard protocol, by applying firm pressure to the prostate (enough to depress the surface) from the base to apex and from the lateral to the median line for each lobe. Exactly three strokes per lobe were performed.

The few drops of prostatic fluid that were discharged upon DRE were collected in a coded container with 4 ml 0.5 M ethylene diamine tetra acetic acid (EDTA). Subsequently, the first voided urine after DRE was collected in two additional coded containers with EDTA.

Following urine collection, the urologist measured the prostate by transrectal ultrasonography and performed a standard 10 core prostate biopsy (five on the left and five on the right side of the prostate) consisting of six laterally directed biopsies and four medially directed biopsies (plus additional biopsies from other areas suspicious for PCa when present). All biopsy cores were treated *lege artis* and examined for the presence of PCa.

The prostatic fluid and urine samples were immediately cooled to 4°C and were mailed in batches with cold packs to our laboratory. The samples were processed within 48 hr after sample acquisition to guarantee good sample quality. These preanalytical procedures are validated [16] and described as standard operating procedure in our laboratory and executed accordingly.

Upon centrifugation at 4°C and 700 g for 10 min, prostatic fluid, and urinary sediments were obtained. These sediments were washed twice with ice-cold, phosphate-buffered saline (PBS) (at 4°C and 700 g for 10 min), snap-frozen in liquid nitrogen, and stored at −70°C. The sediments were spiked with 20 μg of *Escherichia coli* tRNA as a carrier (Roche Diagnostics). Total RNA was extracted from these prostatic fluid and urinary sediments, using TRIzol® Reagent (Invitrogen).

Eight microliters of extracted RNA was dissolved in 2.6 ml of detergent-based stabilization buffer, which lyses the cells and stabilizes the RNA [20].

The quantitative APTIMA® PCA3 test uses the following Gen-Probe technologies: target capture, TMA, and a hybridization protection assay. The components for the APTIMA® PCA3 test include analyte-specific (PCA3 and PSA) target capture reagents, amplification reagents, probe reagents, as well as calibrators, and controls. The APTIMA® PCA3 test was run according to the protocol described earlier by Groskopf et al. [20]. In this assay both the amount of PCA3 RNA and the amount of PSA mRNA in the sample were determined. As the amount of PSA mRNA was used to normalize for the amount of prostate-specific RNA in the samples, the ratio PCA3/PSA RNA was calculated. The PCA3 score was defined as the ratio PCA3/PSA RNA × 1,000.

Of the two samples with copy numbers exceeding the highest calibrator one was diluted 10× in the detergent-based stabilization buffer to bring the sample within the dynamic range of the assay and retested, the other 100×.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 12.0.1 for Microsoft Windows.

To test for differences in PCA3 score between men with a negative and men with a positive biopsy result, we used the two-tailed Mann–Whitney *U*-test. To test for differences in PCA3 score between the prostatic fluid and urine samples in men with a negative biopsy result, as well as to test for differences in PCA3 score between the prostatic fluid and urine samples in men with a positive biopsy result, we used the Wilcoxon signed-rank test. A *P*-value <0.05 was considered significant.

RESULTS

Prostatic fluid and urine samples were collected from 67 men who were to undergo prostate biopsies.

Both samples from all 67 patients arrived in our laboratory cooled at 4°C and were processed within 48 hr of acquisition. All samples were informative (i.e., were positive for PSA mRNA expression).

The mean age of the 67 men in the study population at the time of biopsy was 64.0 (SD 7.2 years), the mean serum PSA value was 8.73 ng/ml (SD 6.61 ng/ml), the mean fraction of free serum PSA (determined in 44/67 = 67%) was 0.19 (SD 0.11) and the mean total prostate volume was 53.1 ml (SD 24.3 ml).

Of the 67 men 23 (34%) had PCa in their biopsies, the remaining had PCa-negative biopsies.

The PCA3 scores for both subject groups obtained from prostatic fluid and urine samples were summarized in a boxplot (Fig. 1).

In prostatic fluid the median PCA3 score for the biopsy negative population was 18 versus 73 for the biopsy positive population. The difference between both groups was highly significant ($P < 0.001$).

In urine the median PCA3 score for the biopsy negative population was 19 versus 48 for the biopsy positive population. Again, the difference between both groups was highly significant ($P = 0.006$).

The difference between the prostatic fluid and urine samples in the biopsy negative population was not significant ($P = 0.21$), neither was the difference between both sample types in the biopsy positive population ($P = 0.19$).

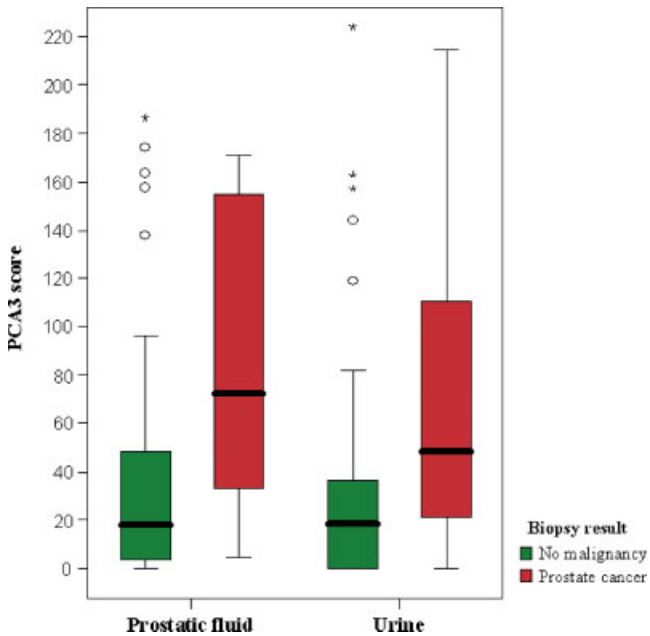

Fig. 1. Boxplot (also known as a "'box-and-whisker diagram") showing the PCA3 scores in prostatic fluid (boxes on the left) and urinary sediments (boxes on the right) after DRE for both men with a negative biopsy result (lighter boxes) and men with a positive biopsy result (darker boxes). The median value (thick black horizontal line), outliers (open circles) and extremes (stars) are shown. The box length is the interquartile range and the "whiskers" extend to 1.5 times this distance. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

The diagnostic efficacy of the APTIMA® PCA3 test on prostatic fluid and urine samples was evaluated by a receiver operating characteristic (ROC) curve in which the test variable was the PCA3 score in both sample types and in which the state variable was the biopsy result (Fig. 2). In the absence of an arbitrary cut-off value, we determined a cut-off value with high specificity and reasonable sensitivity for the diagnosis of PCa. Based on this ROC curve we determined a cut-off value of 66 in prostatic fluid and of 43 in urine. A lower cut-off value would only result in a decrease in the specificity of the test without an increase in the sensitivity. A higher cut-off value would only result in an increase in the specificity of the test at the cost of a considerable decrease in the sensitivity. A similar procedure was described earlier [16]. The area under the curve (AUC), a measure of the diagnostic accuracy of a test, was 0.76 (95% confidence interval (CI) 0.64–0.87) for the APTIMA® PCA3 test on prostatic fluid and 0.70 (95% CI 0.58–0.83) for this test on urine.

For serum PSA the AUC was 0.66 (95% CI 0.53–0.79; Fig. 2). For serum free PSA this was 0.65 (95% CI 0.45–0.85).

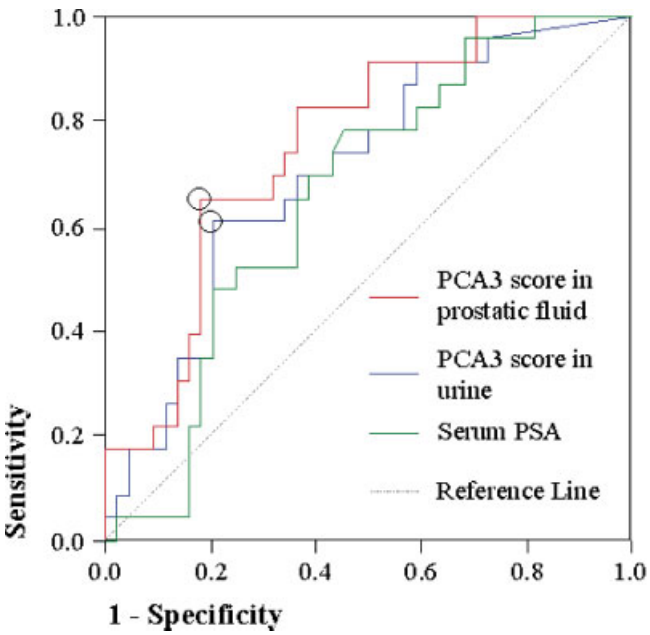

Fig. 2. ROC curve for serum PSA (second line from the bottom) and for the PCA3 score in prostatic fluid (top line) and in urinary sediments (second line from the top) after DRE, in the detection of prostate cancer using biopsy histopathology as standard of reference. The two open circles indicate the determined cut-off values of 66 for the APTIMA® PCA3 test on prostatic fluid and 43 for this test on urine. (PSA, prostate-specific antigen). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Results of the APTIMA® PCA3 Test on Prostatic Fluid Versus the Biopsy Results

	Prostate cancer	No malignancy	Total
PCA3 score >66	15	8	23
PCA3 score <66	8	36	44
Total	23	44	67

Based on the determined cut-off of 66 for the detection of PCa by the APTIMA® PCA3 test on prostatic fluid, the sensitivity was 65%, the specificity was 82%, the positive predictive value was 65% and the negative predictive value was 82% (Table I). For the serum PSA test at the same sensitivity (65%), the specificity was 64% (Fig. 2).

Based on the determined cut-off of 43 for the detection of PCa by the APTIMA® PCA3 test on urine, the sensitivity was 61%, the specificity was 80%, the positive predictive value was 61% and the negative predictive value was 80% (Table II). For the serum PSA test at the same sensitivity (61%), the specificity was 64% (Fig. 2).

To compare the results of the APTIMA® PCA3 test on both sample types, the positive and negative test results were summarized (Table III). Using the determined cut-off values, 57 out of 67 men (85%) were classified as either positive or negative by both tests. Eighteen men were shown to be positive for both tests of whom 12 (67%) had PCa upon biopsy. Both tests were found to be negative for 39 men of whom 33 (85%) had a negative biopsy result.

However, five men were found to be positive for the APTIMA® PCA3 test on prostatic fluid and negative for the APTIMA® PCA3 test on urine. Of these five men, three had PCa upon biopsy. Of the five men who were negative for the APTIMA® PCA3 test on prostatic fluid and were found to be positive for the APTIMA® PCA3 test on urine, two men had PCa upon biopsy.

DISCUSSION

As PSA is not cancer-specific, the widespread use of PSA testing is associated with certain drawbacks, for

TABLE II. Results of the APTIMA® PCA3 Test on Urine Versus the Biopsy Results

	Prostate cancer	No malignancy	Total
PCA3 score >43	14	9	23
PCA3 score <43	9	35	44
Total	23	44	67

TABLE III. Discordance Analysis of the APTIMA® PCA3 Test on Prostatic Fluid Versus on Urine Using the Determined Cut-Off Values

	PCA3 score in urine >43	PCA3 score in urine <43	Total
PCA3 score in prostatic fluid >66	18 (12 PCa)	5 (3 PCa)	23
PCA3 score in prostatic fluid <66	5 (2 PCa)	39 (6 PCa)	44
Total	23	44	67

example, a high negative biopsy rate and the diagnosis of clinically irrelevant tumors (overdiagnosis) with the danger of overtreatment. As a result, there is an urgent need for PCa-specific markers that can improve the specificity in PCa diagnosis and can differentiate between indolent and aggressive disease.

The APTIMA® PCA3 test on urine after DRE has been shown to improve specificity in PCa diagnosis, [16,18–20] which could be applied to prevent unnecessary prostate biopsies. In this exploratory study we evaluated the APTIMA® PCA3 test on prostatic fluid and compared it with the APTIMA® PCA3 test on urine in a clinical research setting.

The results indicate that the APTIMA® PCA3 test performs comparably on prostatic fluid and on urine after DRE. The AUC was 0.76 for the APTIMA® PCA3 test on prostatic fluid and at a determined cut-off value of 66, the sensitivity for the detection of PCa was 65%, the specificity was 82% and the negative predictive value was 82%. The AUC was 0.70 for the APTIMA® PCA3 test on urine and at a determined cut-off value of 43, the sensitivity for the detection of PCa was 61%, the specificity was 80% and the negative predictive value was 80%. These results compare favorably with the results obtained thus far in other studies using PCA3 gene-based analysis (Table IV).

For reason of comparison we showed that at an equal sensitivity of either 61 or 65%, the specificity for the serum PSA test was only 64%, compared to 82% for the APTIMA® PCA3 test on prostatic fluid and 80% for this test on urine. This suggests that the APTIMA® PCA3 test on either sample type can be used to overcome the current problems with PSA testing, by improving specificity in PCa diagnosis to reduce the number of unnecessary biopsies and thereby the number of cancers detected serendipitously.

The differences between both sample types can be subdivided according to category. In the biopsy negative population there was hardly any difference in the median PCA3 score between the prostatic fluid and urine samples (18 vs. 19). However, in men with PCa there was a difference (although not significant) in the median PCA3 score between the prostatic fluid and urine samples (73 vs. 48). This difference was not caused by a higher amount of PCA3 RNA in the prostatic fluid samples, but by a median 1.4-fold increase in the amount of PSA mRNA in the urine samples compared with the prostatic fluid samples (data not shown). This may indicate that in urine samples the relative fraction of non-malignant prostate epithelial cells is higher. Moreover, the levels of PSA mRNA in the urine samples of men with PCa showed a significant 2.2-fold increase over the amount of PSA mRNA in the urine samples of men with negative biopsies (data not shown). This might be a mere reflection of increased cellularity in cancer specimens.

In addition, there is no obvious explanation for the discordance between the results of the APTIMA® PCA3 test on both sample types as seen in 10 men (of whom five had PCa upon biopsy). An explanation could be that in cases in which the prostatic fluid sample tested positive and the urine sample did not, the prostatic fluid sample contained the majority of the

TABLE IV. The Performance in Prostate Cancer Diagnosis of PCA3 Gene-Based Analysis Following DRE

PCA3 test method	Sample type	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Area under the ROC curve	Reference
TRF-based	Urine	67	83	90	0.72	[16]
uPM3™	Urine	66	89	84	0.86	[18]
uPM3™	Urine	82	76	87	0.87	[19]
APTIMA®	Urine	69	79	89	0.75	[20]
APTIMA®	Urine	61	80	80	0.70	This study
APTIMA®	Prostatic fluid	65	82	82	0.76	This study

mobilized cells with PCA3 overexpression, that is, PCa cells, leaving only a minor fraction for the urine sample. Which would result in a higher PCA3 score in the prostatic fluid sample. Vice versa, in cases in which the urine sample tested positive and the prostatic fluid sample did not, the prostatic fluid sample could have contained only a minor fraction of the mobilized cells with PCA3 overexpression, leaving the majority for the urine sample. Which would result in a lower PCA3 score in the prostatic fluid sample.

In this study, we used the sediments of prostatic fluid and urine samples for the APTIMA® PCA3 test instead of whole urine as was described by Groskopf et al. [20]. A possible advantage of using the sediment of a urine sample may be that the size of the sample is increased and that the clinical correlation is linked to cells and cell fragments in the sediment. The test on whole urine only analyzes 2 ml of the total sample. In contrast, analyzing the sediment is equal to analyzing all the cells in the whole sample, as these are concentrated in the sediment. Our results indicate that the analysis of prostatic fluid sediments is feasible and provides comparable clinical utility to urine specimens. A combination of the two specimens may be expected to yield higher sensitivity and specificity.

Other PCa Markers in Prostatic Fluid

To our knowledge, thus far only five PCa markers have been investigated in prostatic fluid: ornithine decarboxylase (ODC) [7], free insulin-like growth factor-1 (IGF-1) [9], telomerase activity/expression [4,11,12], hypermethylation of the glutathione S-transferase P1 (GSTP1) promoter [4] and dipeptidylpeptidase IV (DPIV) [13].

Most studies however, were hampered by a limited sample size [7,12] and/or studied a case mix of men (with and without PCa) instead of the standard clinical population in which PCa is suspected [7,9,11,12]. One study (of IGF-1) showed a poor clinical correlation [9]. Other studies emphasized the need to perform an extensive prostate massage under general anesthesia [11,12] or ''urethral milking'' [4], which would prohibit the use of the test in a routine clinical setting. But most importantly, it should be noted that none of the studies used standardized, validated methods performed on a routine molecular diagnostic test platform.

In our study of prostatic fluid specimens, we have shown that this procedure *can* be performed in a routine clinical setting. Furthermore, we have utilized a test that has been validated in different laboratories, performed on a routine molecular diagnostic test platform which is suitable and ready for clinical implementation.

CONCLUSIONS

To our knowledge, we are the first to examine the diagnostic value of the APTIMA® PCA3 test using prostatic fluid samples. The results of our exploratory study suggest that the clinical utility of this test using prostatic fluid is comparable to that using urine after DRE and that both tests can be used to overcome the current problems with PSA testing by improving specificity in PCa diagnosis. A combination of the two specimens may be expected to yield higher sensitivity and specificity. This implies that the APTIMA® PCA3 test can be performed not only on urine, but also on prostatic fluid, further expanding the applicability of PCA3 gene-based analysis using alternative collection methods.

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