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Numeric Definition of the Clinical Performance of the Nested Reverse Transcription-PCR for Detection of Hematogenous Epithelial Cells and Correction for Specific mRNA of Non-Target Cell Origin as Evaluated for Prostate Cancer Cells

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Background: **Inappropriate quality management of reverse transcription-PCR (RT-PCR) assays for the detection of blood-borne prostate cancer (PCa) cells hampers clinical conclusions. Improvement of the RT-PCR methodology for prostate-specific antigen (PSA) mRNA should focus on an appropriate numeric definition of the performance of the assay and correction for PSA mRNA that is not associated with PCa cells.**

Methods and Results: **Repeated (RT-)PCR tests for PSA mRNA in single blood specimens from PCa patients and PCa-free controls, performed by four international institutions, showed a large percentage (≈50%) of divergent test results. The best estimates of the mean, λ (SD), of the expected Poisson frequency distributions of the number of positive tests among five replicate assays of samples from PCa-free individuals were 1.0 (0.2) for 2 × 35 PCR cycles and 0.2 (0.1) for 2 × 25 PCR cycles. Assessment of the numeric value of the mean can be considered as a new indicator of the performance of a RT-PCR assay for PSA mRNA under clinical conditions. Moreover, it determines the required number of positive test repetitions to differentiate between true and false positives for circulating prostate cells. At a predefined diagnostic specificity of ≥98%, repeated PCRs with λ of either 1.0 or 0.2 require, respectively, more than three or more than one positive tests to support the conclusion that PSA mRNA-containing cells are present.**

Conclusions: **Repeated nested PCR tests for PSA and appropriate handling of the data allow numeric quantification of the performance of the assay and differentiation between analytical false and true positives at a predefined accuracy. This new approach may contribute to introduction of PSA RT-PCR assays in clinical practice.**

Adenocarcinoma of the prostate (PCa)[6] is one of the most common malignancies and the second leading cause of male cancer deaths in Western populations *(1)*. Despite improvement of treatment modalities for locally confined disease, a subset of patients suffer recurrence attributable to locally progressive disease or distant metastases *(2)*. The latter, representing 25–40% of all patients, stresses the necessity of additional, accurate staging methods focusing on early detection of subclinical micrometastatic cell dissemination *(2, 3)*.

Measurement of prostate-specific antigen (PSA) mRNA transcripts by reverse-transcription-PCR (RT-PCR) has been introduced as a means to identify extra-

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[6] Nonstandard abbreviations: PCa, prostate cancer; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; iPSA, illegitimate PSA; BPH, benign prostatic hyperplasia; PBS, phosphate-buffered saline; and PBMNC, peripheral blood mononuclear cell.

capsular PCa cells in blood, lymph nodes, and bone marrow *(4, 5)*. Clinical studies have addressed the sensitivity and specificity of (nested) RT-PCR assays for PSA mRNA for molecular staging. Nevertheless, the clinical value of the RT-PCR methodology in PCa remains controversial. The reported sensitivity of detection of blood-borne PCa cells in patients with confirmed metastatic disease ranges from 25% to 80%, whereas the correlation between pathologic staging and PSA RT-PCR results was inconsistent and contradictory *(4, 5)*.

The high rate of contradictory PSA RT-PCR results has been attributed to a series of factors associated with the large variety of applied RT-PCR procedures and/or patient selection. These factors include differences in the preanalytical work-up of RNA, the analytical sensitivity of the applied RT-PCR procedures, reduced concentrations or absence of circulating PCa cells in some patients with confirmed metastatic disease, patient-dependent differences in PSA mRNA expression per circulating cell, and interference by, among others, illegitimate PSA (iPSA) mRNA and PSA mRNA from circulating nonmalignant prostate cells *(6–10)*. These and inherent, essentially random, properties of the RT-PCR process under certain conditions *(5, 11–13)* require methodic analyses of the various sources of intraassay and interlaboratory variation *(11, 14, 15)*.

An international consortium initiated a study of the intraassay and interlaboratory variation of a nested RT-PCR assay for PSA mRNA. This goal required the development of a numeric definition of the performance of the RT-PCR assay under clinical conditions, determination of the necessary number of test repetitions, and correction for interference for, among other factors, iPSA mRNA transcripts.

Materials and Methods

CELL CULTURE

The human lymph node-derived, constitutively PSA-expressing PCa cell line LNCaP *(16)*, obtained from the American Type Culture Collection, was used as a positive control for the PSA RT-PCR assay. Stock cell lines cultures were cultured as described previously *(17)* and were screened routinely for *Mycoplasma*.

PARTICIPANTS AND BLOOD SAMPLING

Four institutions (institution 1, Hieronymus Bosch Hospital, Den Bosch, The Netherlands; institution 2, University of Amsterdam, Amsterdam, The Netherlands; institution 3, University of Leuven, Leuven, Belgium; institution 4, University of Aachen, Aachen, Germany) studied 76 individuals, including apparently healthy volunteers [12 females (3 at institution 1, 3 at institution 2, 3 at institution 3, and 3 at institution 4) and 12 males (3 at institution 1, 4 at institution 2, 3 at institution 3, and 2 at institution 4)], 11 patients with benign prostatic hyperplasia (BPH; 2 at institution 1, 3 at institution 2, 3 at institution 3, and 3 at institution 4), and 41 patients with localized PCa (10 at institution 1, 11 at institution 2, 10 at institution 3, and 10 at institution 4). The diagnosis of BPH was based on histologic analysis of tissue obtained by transurethral resection. The PCa patients represented a heterogeneous group of staged at or below pT3N0M0. No particular effort was made to stratify the participants into well-defined groups with respect to stage because this was not the aim of this study. All PCa patients underwent radical prostatectomy.

Blood specimens were collected from an antecubital vein 1 day preoperatively and at least 3 weeks after digital rectal examination or prostate needle biopsy. Blood (6–7 mL) was routinely collected in 10-mL EDTA-containing Vacutainer Tubes (Becton Dickinson).

PROCESSING OF BLOOD SPECIMENS

Blood specimens (5 mL) were mixed with an equal volume of ice-cold phosphate-buffered saline (PBS; cat. no. 14190-094 Gibco BRL, Life Technologies). We fractionated 5 mL of the mixture twice with use of 4-mL Ficoll-Paque gradients (Amersham Pharmacia Biotech) and centrifugation at 1000*g* for 20 min at 4 °C. After collecting the peripheral blood mononuclear cell (PBMNC)-containing interphases, we pooled the cells and washed them twice in PBS by centrifugation at 400*g* for 10 min at 4 °C. We subsequently suspended the cells in 1 mL of PBS and determined cell yield ($\sim 1 \times 10^6$ PBMNC/mL of blood). After centrifugation at 4500*g*, the pellet was either processed immediately for RNA extraction or dissolved in TRIzol® (Gibco BRL, Life Technologies), stored at −20 °C, and processed within 1 month.

The cell pellet was lysed with 1 mL of TRIzol, and RNA was prepared according to the manufacturer's recommendations. The yield of RNA was quantified by spectrophotometric analysis at 260 nm, and RNA quality was estimated from the ratio of the ultraviolet absorbance at 260 and 280 nm. Extracted RNA was stored at −20 °C until the RT-PCR test was performed (within 1 month).

RT-PCR

cDNA was reverse-transcribed from 5 μg of total RNA in a reaction volume of 20 μL containing 4.0 μL of 5× First Strand Buffer, 2.0 μL of 0.1 M dithiothreitol, 1.0 μL of a dinucleotide triphosphate mixture (10 mM each of dATP, dCTP, dGTP, and dTTP), 1.0 μL of 0.5 g/L oligo$(dT)_{12–18}$ primers, and 0.4 μL of 200 U/μL Superscript™, RNase H^- Reverse Transcriptase. All reagents were obtained from Gibco BRL, Life Technologies. The reaction was performed according to the manufacturer's recommendations (Superscript; cat. no. 18064-014), i.e., incubation for 1.5 h at 42 °C and inactivation at 70 °C for 15 min. cDNA was stored at −20 °C or used immediately for PCR amplification.

The PCR for PSA mRNA was performed according to a modified procedure described previously by Israeli et al. *(17)*. cDNA was amplified by nested PCR. In the first reaction, 2 μL of cDNA was added to 50 μL of PCR

mixture containing 5 μL of 10× PCR buffer (0.1 M Tris-HCl, 0.5 M KCl, pH 8.3), 1 μL of dinucleotide triphosphate mixture (10 mM each), 3 μL of 25 mM $MgCl_2$, 0.2 μL of 5 U/μL AmpliTaq® DNA Polymerase (Perkin-Elmer), and 1.0 μL each of the (HPLC-purified) outer primers (10 μM; BioSource). The outer primer set has been described previously *(17)*. Verification of the nucleotide sequence by the BLASTN 1.4.9MP alignment search tool, however, revealed a mismatch in the nucleotide sequence of the upstream primer. Accordingly, the original primer (5′-TACCCACTGCATCAGGAACA-3′) was replaced by the corrected primer (5′-TGCCCACTGCATCAGGAACA-3′). PCR was carried out with locally available thermocyclers with the following cycling conditions: 1 cycle of 95 °C for 3 min; 35 cycles of 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 20 s; and 1 cycle of 72 °C for 7 min. Reaction mixtures were then stored at 4 °C overnight.

In the nested PCR, 2.0 μL of the first reaction mixture was used as template for an identical PCR reaction with the inner primers described by Israeli et al. *(17)*. The outer and inner primer sets yielded 486- and 355-bp PCR products, respectively. In an additional series of experiments, the PCR was performed with 25 × 2 cycles. The 100-bp ladder was obtained from Amersham Pharmacia Biotech.

The PCR products were fractionated by electrophoresis in 2% agarose gels containing ethidium bromide. Gels were visualized by ultraviolet light and photographed with Polaroid 665 film or recorded with the Gel Doc 1000 System (Bio-Rad), depending on the locally available equipment.

Negative and positive controls were included in each series of RT-PCR tests for PSA mRNA. Positive controls consisted of RNA isolated from LNCaP cells. In the negative controls, water replaced the cDNA sample and/or the reverse transcriptase was omitted from the reverse transcription step. To assess the integrity of RNA extracted from the blood specimens, cDNA regions of the ubiquitous housekeeping genes β_2-microglobulin and/or β-actin, obtained in the above-described reverse transcription reaction, were amplified in a conventional, single PCR *(17)*. The appropriate primers were obtained from BioSource.

STATISTICS

The significance of relationships was evaluated by the Student *t*-test for comparison of quantitative variables and the χ^2 test, Fisher exact test, or a simulated exact test for categorical variables. Accepting, in the current study, the four participating institutions as "one center" and accordingly pooling the results of the institutions, we assessed the intraassay variation of the PSA PCR protocols by treating the frequency distributions of PCa-free individuals and PCa patients with positive test results in replicate PCR tests on single blood specimens as a Poisson probability density function:

$$P(x) = (e^{-\lambda}\lambda^x)/x!$$

and cumulative Poisson probability function:

$$P(\leq x) = \sum_{k=0}^{x} (e^{-\lambda}\lambda^x/x!)$$

in which x is the number of positive events (positive PSA PCR test results) and λ is the best estimate of the mean number of events. The Poisson probability density function provides the number of exactly x-occurring positive events, whereas the cumulative Poisson probability function provides the number of events between 0 and x inclusive. The expected number of more than x events, $P(>x)$, equals $1 - P(\leq x)$. Goodness of fit was assessed by the *G*-test. The applied software packages were SPSS for Windows (release 10.0.7), S-Plus, and the add-in program XLSTAT 4.4 for Microsoft® Excel 97 (www.xlstat.com). $P < 0.05$ was considered statically significant.

Results

INTERLABORATORY VARIATION AND DETECTION LIMIT OF PSA RT-PCR

To gain initial insight into the analytical sensitivity and interlaboratory variation of the RT-PCR assay for PSA mRNA, batches of reversed-transcribed mRNA (cDNA) isolated from serially diluted LNCaP cells (1–1000 cells/mL of female blood) were prepared at one institution, shipped, and analyzed by all four participating institutions. Despite differences in thermocyclers, gel electrophoresis instrumentation, and gel visualization systems, the common PCR (protocol, 35 × 2 cycles) method for PSA detected 1 LNCaP cell/mL of blood (1 LNCaP cell per 10^6–10^7 PBMNCs) at each of the four participating sites.

VARIATION AND NUMERIC DEFINITION OF NESTED PCR FOR PSA cDNA AMPLIFICATION

In an initial series of experiments, the intraassay or tube-to-tube variation of the nested PCR assay for PSA was studied using samples from patients with histologically confirmed PCa. As illustrated in Fig. 1, for triplicate PCR amplifications of PSA cDNA, PCR test results for single blood specimens showed considerable variation. We observed consistent positive, consistent negative, and divergent (revealing both positive and negative) PCR test results. It should be noted that the repeated PCR tests for both β-actin and β_2-microglobulin mRNA, which encodes for housekeeping proteins, invariably produced consistent amplification test results (results not shown). Negative water controls and controls without reverse transcriptase were always negative during repeated testing.

Subsequently, the recruiting institutions and their associated laboratories extended the experiments with a collective RT-PCR procedure for PSA mRNA involving fivefold, simultaneously repeated PCR tests on single blood specimens from a series of patients with confirmed

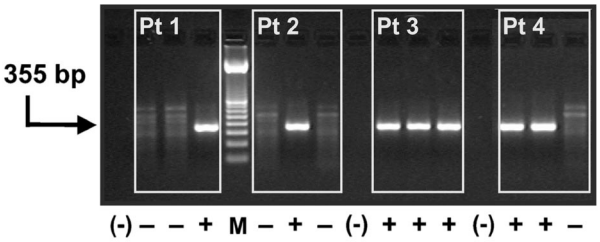

Fig. 1. Representative illustration of the intraassay variation of the PSA RT-PCR assay performed on single blood specimens from PCa patients, obtained by one of the participants.

Reverse-transcribed mRNA of patients (*Pt*) with PCa was simultaneously amplified [(35 × 2-cycle) protocol], in triplicate. +, positive (355-bp band) results; −, negative results. Results for patients with consistently negative test results are not shown. *Lane M*, 100-bp ladder; *Lane (−)*, H_2O control. Note the nonspecific PCR products in the 355-bp-negative lanes, likely representing the "PCR paradox" *(20)*.

PCa and from PCa-free individuals. As shown in Table 1, the distribution of results for a series of 41 PCa patients with consistently positive, consistently negative, and divergent PSA PCR test results was independent ($P = 0.099$) of the recruiting institution. Pooling of results from the institutions showed that, in this group of nonstratified patients, the results of the repeated PSA PCR tests were consistent (either positive or negative) in 54% and inconclusive in 46% of the cases (Table 1).

These observations suggested that low-abundance PSA mRNA transcripts were detected randomly and that stochastic factors, affecting the process of PSA cDNA amplification were involved. This highlights the importance of repetitive testing. Moreover, positive test results may originate from PSA mRNA transcripts of non-prostate cell origin or leukocytes, iPSA mRNA, and/or circulating nonmalignant prostate cells. Therefore, translation of inconclusive RT-PCR test results for PSA mRNA into clinically relevant conclusions requires extensive characterization of the performance of the PSA RT-PCR assay under clinical conditions and quantification of the degree of interference by PSA mRNA from cells other than PCa cells.

Table 1. Results obtained at four institutions applying equal, multiple nested PCR tests for PSA cDNA in single blood specimens from patients with PCa.[a]

	No. (relative frequency) of patients with consistent positive, consistent negative, and inconsistent PSA PCR test results			
Institution	Consistent positive	Consistent negative	Inconsistent	Total
1	2 (20%)	2 (20%)	6 (60%)	10
2	5 (46%)	0 (0%)	6 (54%)	11
3	2 (20%)	5 (50%)	3 (30%)	10
4	1 (10%)	5 (50%)	4 (40%)	10
Total	10 (24%)	12 (29%)	19 (46%)	41

[a] cDNA, reverse transcribed from mRNA processed from a single blood specimen from each patient, was analyzed fivefold simultaneously. The results were categorized according to consistent positive, consistent negative, and inconsistent (at least one positive and one negative test) PCR test repetitions. Comparison between the institutions: $\chi^2 = 10.68$; $df = 6$; $P = 0.099$.

Acepting BPH patients and healthy males and females as being without blood-borne cells of prostate origin, we performed fivefold PSA PCR tests on single blood specimens from these individuals (Table 2). Contrary to the results obtained for PCa patients, we found no consistent positive PSA PCR test repetitions within this PCa-free group. However, comparison between PCa-free individuals and PCa patients revealed no differences ($P = 0.630$) between the occurrence of consistent negative and divergent PCR repetitions in the two groups. In subsequent analyses, BPH patients and healthy males and females were considered as one group because the distribution of PSA PCR test results was independent ($P = 0.272$) of the type of individual (Table 2). In 18 of 35 individuals (51%), at least one positive PSA PCR test result was seen; this was similar to the rate (46%; 19 of 41) of inconsistent test results for PCa patients. Within the PCa-free group (n = 35), the observed numbers of individuals with zero, one, two, three, four, and five positive tests for the quintuplicate assays were 17 (49%), 8 (23%), 4 (11%), 4 (11%), 2 (6%), and 0 (0%), respectively (Table 2). The probability of low-abundance PSA mRNA transcripts was assessed with use of the Poisson distribution, which revealed the expected frequency distribution of positive PCR tests. In the PCa-free group, the fitted ($P < 0.05$) Poisson frequency distribution yielded an estimate of the mean or density parameter, λ (SD), of 1.0 (0.2) positive test results (Fig. 2A). This value ($\lambda = 1.0$) predicts that at least one positive PCR test result from among five results, presumably representing iPSA mRNA and/or PSA mRNA from healthy prostate cells, can be expected with a probability [$P(>0)$] of 63% (second column in Table 3). These observations indicate a high rate of random detection of blood-borne PSA mRNA transcripts in PCa-free individuals, i.e., a high rate of clinical false positives. Most importantly, however, the probability of four or more positive test results [$P(>3)$] of a total of five results on a single blood specimen is only ≈2% (second column in Table 3).

The performance of the PSA nested PCR protocol on single blood specimens from patients with confirmed PCa was evaluated in a similar manner. The observed frequencies distribution for 41 patients with zero, one, two, three, four, and five positive test results were 12 (29%), 6 (15%), 5 (12%), 4 (10%), 4 (10%), and 10 (24%), respectively (Table 2). This frequency distribution presumably reflects detection of PSA mRNA transcripts of PCa cell origin and other PSA mRNA transcripts, such as iPSA mRNA. This heterogeneous character of a PCa patient population complicates the statistical, numeric evaluation of the performance of the PSA PCR assay.

Anticipating the results obtained for the PCa-free group, we evaluated the performance of the PSA PCR assay for blood specimens from patients after omitting the test replications with four and five positive PSA PCR test

Table 2. Intraassay variation of nested PCR for PSA cDNA in blood specimens from patients with PCa or BPH and from healthy female and male volunteers.[a]

	No. (%) of individuals with consistent positive, consistent negative, and inconsistent PSA PCR results within five repeated PSA PCR tests.							
			Inconsistent					
	Consistent positive (five[b])	Consistent negative (zero)	One	Two	Three	Four	Total[c]	Total
BPH	0 (0%)	5 (46%)[d,e]	1 (9%)	2 (18%)	1 (9%)	2 (18%)	6 (54%)	11
Male	0 (0%)	6 (50%)	2 (17%)	2 (17%)	2 (17%)	0 (0%)	6 (50%)	12
Female	0 (0%)	6 (50%)	5 (42%)	0 (0%)	1 (8%)	0 (0%)	6 (50%)	12
PCA	10 (24%)	12 (29%)	6 (32%)	5 (26%)	4 (21%)	4 (21%)	19 (46%)	41
BPH + males + females	0 (0%)	17 (49%)	8 (23%)	4 (11%)	4 (11%)	2 (6%)	18 (51%)	35

[a] cDNA, reverse transcribed from mRNA processed from a blood specimen from each individuals, was analyzed five times simultaneously.

[b] Number of positive PSA PCR test results for single blood specimens.

[c] Total number and percentages of individuals with inconsistent PSA PCR test results.

[d] Comparison between PCa-free individuals based on the consistent negative and inconsistent, categorized PCR test results: $\chi^2 = 9.906$; $df = 8$; $P = 0.272$.

[e] Comparison between BPH patients, healthy males and females, and PCa patients based on the consistent negative and inconsistent, categorized PSA PCR test results: $\chi^2 = 9.843$; $df = 12$; $P = 0.630$.

results. As illustrated in Fig. 2B, the estimated density parameter of the accordingly fitted ($P < 0.05$) Poisson frequency distribution was 1.0 (0.2) positive test results. The equal expected frequency distributions for PCa patients and PCa-free individuals indicated that the performance of the applied nested PSA PCR was not affected by the source of the blood specimens, PCa-free individuals or PCa patients. If we accepted a diagnostic specificity of ≥98% as necessary for diagnostic purposes, the expected Poisson frequency distributions suggested strongly that four or more positive PSA PCR test results represent the presence of blood-borne PCa cells, i.e., clinically true positives. This means that within the investigated group of 41 nonstratified patients, the present, high-sensitivity RT-PCR assay for PSA mRNA detected 14 (34%) patients with blood-borne PSA mRNA transcripts, presumably originating from prostate (cancer) cells (Fig. 2B).

This observed intraassay variation for repeated PSA PCR tests on clinical blood specimens seemed to be in accordance with variations found for highly diluted LNCaP PSA cDNA. As observed by one of the participants, simultaneously repeated PCR tests (35 × 2 cycles protocol) on serially diluted LNCaP cDNA showed divergent test results with positive/negative ratios of 5/0, 3/2, 1/4, and 0/5 at dilutions of $1{:}10^5$, $1{:}10^6$, $1{:}10^7$, and $1{:}10^8$, respectively.

TEST RESULTS WITH PSA PCR PROCEDURES OF DIFFERENT SENSITIVITIES

The use of PSA RT-PCR procedures with an appropriately adjusted sensitivity has been suggested to prevent misinterpretation of results for blood specimens from PCa patients. For this reason, we compared the (35 × 2)-cycle PSA PCR procedure with a PSA PCR procedure that incorporated a (25 × 2)-cycle protocol but was otherwise identical. Serially diluted LNCaP cells indicated that the (analytical) sensitivity (lower detection limit) of the (25 × 2)-cycle PSA PCR procedure was lower by approximately one log unit. Application of the (25 × 2)-cycle procedure to blood specimens from 14 randomly selected PCa-free individuals, representing a total of 70 PSA PCR tests, produced a total of 3 (4%) positive PSA PCR test results. Detailed analysis revealed an expected Poisson frequency distribution characterized by a λ (SD) of 0.2 (0.1) positive test results for the (25 × 2)-cycle procedure (Fig. 3A). The corresponding best estimate of the mean for the (35 × 2)-cycle PCR procedure was 1.0 (0.3) (Fig. 3B). From these expected frequency distributions, we deduced that the probability of detection of one or more positive tests was 18% with the (25 × 2)-cycle procedure (λ = 0.2; third column in Table 3), whereas the corresponding probability for the (35 × 2)-cycle procedure (λ = 1.0) was 63%. Compared with the (35 × 2)-cycle PSA PCR procedure, these data showed a considerable decrease in the number of false-positive test results when the (25 × 2)-cycle PSA PCR procedure was performed.

To verify whether the advantage of reduced detection of iPSA mRNA transcripts was accompanied by decreased diagnostic sensitivity, we analyzed blood specimens from 29 randomly selected patients with confirmed PCa with both the (25 × 2)-cycle and (35 × 2)-cycle PSA PCR procedures (Fig. 3, C and D). An initial, superficial analysis of the obtained test results showed that the (25 × 2)-cycle procedure gave four or five positive test results for 6 (21%) patients compared with 11 (38%) patients analyzed with the (35 × 2)-cycle procedure. These results suggested that reduction of the analytical sensitivity of the PSA RT-PCR assay (increased detection limit) decreased the diagnostic sensitivity. For this conclusion, we assumed that four or more positive test results (within repeated 35 × 2 PCR tests) represented true positives (Fig. 3, B and D). The expected Poisson frequency distribution [λ = 0.2 (0.1)] for a (25 × 2)-cycle PSA PCR procedure predicted, however, that even two or more positive tests

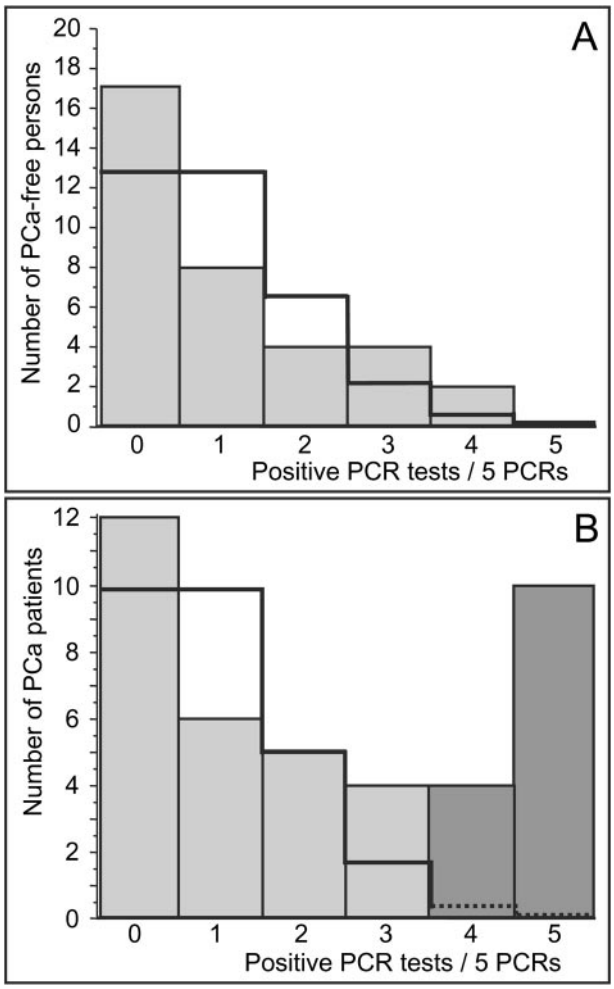

Fig. 2. Observed and expected frequency distributions of PCa-free individuals and PCa patients with positive PCR-PSA test results within fivefold test repetitions [(35 × 2)-cycle protocol], performed on single blood specimens and pooled for the four participating institutions.

The *columns* represent the observed frequencies. (*A*), PCa-free individuals. The fitted ($G = 7.498 < \chi^2_{0.05[3]} = 7.815$; n = 35) expected Poisson frequency distribution (*solid line*) showed an estimated sample mean [λ (SE)] of 1.0 (0.2) positive PCR test results. (*B*), PCa patients. Omitting those patients with four and five positive PSA PCR test results (*dark gray columns*; n = 14), we calculated an expected Poisson frequency distribution ($G = 5.580 < \chi^2_{0.05[2]} = 5.991$; n = 27) with a λ of 1.0 (0.2) positive PCR test results (*solid line*). The extrapolated *dashed line* indicates the expected frequencies of four and five positive PSA PCR test results, according to a Poisson distribution with λ = 1.0.

per blood specimen should be considered as true positives with a specificity [$P(>1)$] of ~98% (Fig. 3, A and C; third column in Table 3). This refined analysis revealed that nine (31%) blood specimens from patients with confirmed PCa, analyzed with the 25 × 2 PCR protocol, may be considered as positive for the presence of blood-borne PCa cells (Fig. 3C). Therefore, appropriate analysis indicated that a considerable reduction of interference by iPSA mRNA transcripts, i.e., increase in clinical specificity, could be accomplished by reduction of the sensitivity of a PSA RT-PCR assay without a major loss of diagnostic sensitivity.

It is of interest to note that, compared with the high analytical sensitivity (35 × 2 cycles) PSA PCR assay, the less sensitive (25 × 2 cycles) assay required fewer (two to three) test repetitions to detect true positives with an identical diagnostic sensitivity.

Discussion

The authors of several studies have indicated a correlation between PSA RT-PCR tests for detection of blood-borne PCa cells and pathologic stage, whereas the authors of other studies reported contradictory results *(4–8, 17)*. It seems reasonable to speculate that the controversy surrounding the clinical utility of the assay originates from the large variety of PSA PCR protocols. The analytical sensitivity (lower detection limit) of the clinically applied PSA RT-PCR assays, generally defined as the number of LNCaP cells per PBMNC, ranged from 1 LNCaP cell in 10^5 PBMNCs to 1 in 10^8 *(4, 5, 7, 9, 17, 18)*. The value of LNCaP cells as a comparative calibrator is, however, limited because of considerable variation in the number of PSA mRNA copies per LNCaP cell, even when cultured under identical conditions *(19)*. Moreover, inherent stochastic characteristics associated with RT-PCR procedures that affect the final test result can not be underestimated. These characteristics include the possibility of sampling of target mRNA and target cDNA (sampling error) *(11, 13)*, the probability of primer annealing to low-abundance target cDNA within a complex mixture of cDNA molecules (Monte Carlo effect) *(12)*, and the tolerance of PCR to mismatches and amplification of nontarget templates in an unpredictable manner (PCR paradox) *(20)*. Because of the nonlinear, exponential character of the cDNA amplification process, small, often inevitable differences in the initial conditions, such as the technical specifications of the thermocycler, may affect the final yield of PCR product in a considerable and unpredictable manner *(5, 11, 21)*. In accordance with previous studies *(9, 11)*, our extensive, multifold test procedure supported the occurrence of unpredictable, inconsistent PSA PCR test results for blood specimens from both PCa-free individuals and PCa patients. These observations are of signifi-

Table 3. Probabilities of $>x$ [$P(>x)$] positive PSA PCR test results of with different Poisson parameter densities (λ).[a]

	$P(>x)$,[b] %	
No. of positive tests (x)	**λ = 1.0**	**λ = 0.2**
0	63.2	18.1
1	26.4	1.8
2	8.0	0.1
3	1.9	<0.1
4	0.4	<0.1

[a] x represents the number of positive test results for repeated PSA PCR tests. The probabilities of $>x$ [$P(>x)$] positive test results were obtained from Poisson tables or calculated as described in the statistics section of *Materials and Methods*.

[b] The presented probabilities are for the expected Poisson distributions with the indicated density parameters (λ).

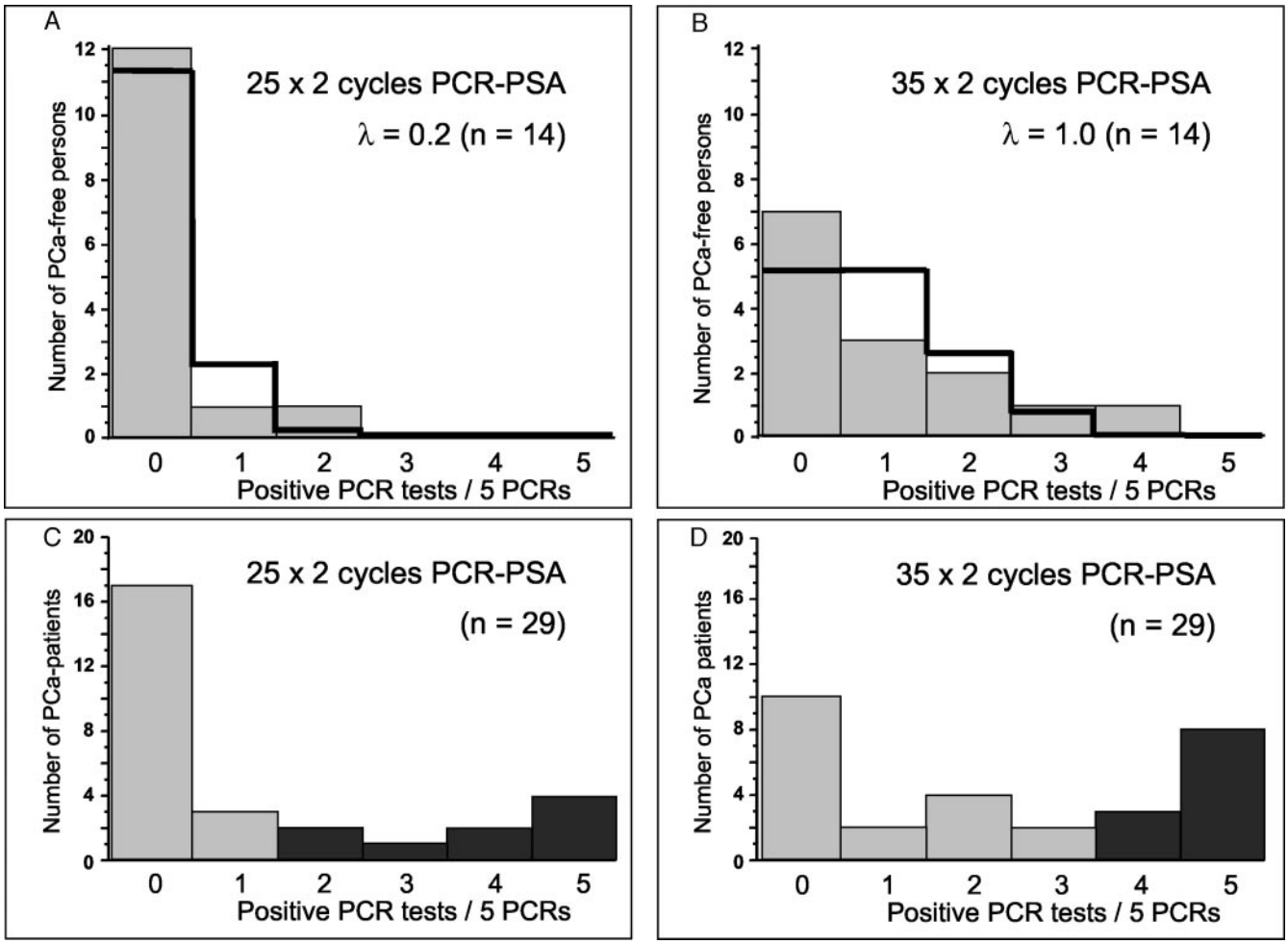

Fig. 3. Observed and expected frequency distributions of PCa-free individuals and PCa patients with indicated positive PSA PCR test results after fivefold test repetitions with the (25 × 2)- and (35 × 2)-cycle PCR procedures.

The analyzed populations were a randomly selected part of the total cohort of PCa-free individuals and PCa patients. The *columns* represent the observed frequencies. (*A*), frequency distributions of blood specimens from PCa-free individuals, analyzed with the (25 × 2)-cycle PCR procedure. *Solid line*, fitted ($G = 2.332 < \chi^2_{0.05[1]} = 3.841$; n = 14) expected Poisson frequency distribution with λ = 0.2 (0.1) positive PCR test results. (*B*), frequency distributions of blood specimens of PCa-free individuals, analyzed with (35 × 2)-cycle PCR procedure. *Solid line*, fitted ($G = 3.124 < \chi^2_{0.05[3]} = 7.815$; n = 14) expected Poisson frequency distribution with λ = 1.0 (0.3) positive PCR test results. (*C*), frequency distributions of blood specimens of PCa patients (n = 29), analyzed with the (25 × 2)-cycle PCR procedure. (*D*), frequency distributions of blood specimens from PCa patients (n = 29), analyzed with the (35 × 2)-cycle PCR procedure.

cance for the development of a standardized assay, a frequently raised topic *(4, 5)*, because even a technically equal assay does not exclude intraassay and interlaboratory variations.

Another setback for the clinical application of the RT-PCR assay for PSA mRNA came to light during attempts to improve assay sensitivity. It became evident that PSA mRNA synthesis is not restricted to epithelial prostate cells. PSA transcripts appear to be present in nonprostatic healthy and tumor tissues and cell lines and, most importantly, in PBMNCs from healthy males and females *(9, 10)*. Compared with other studies, our study revealed a high (51%) rate of such clinical false positives in PCa-free individuals, possibly because of a smaller "sampling error" provided by the multifold testing. Confirming the conclusions of Henke et al. *(10)*, these data provide comprehensive evidence that increased sensitivity for a PSA RT-PCR assay is accompanied by a decrease in diagnostic specificity.

Taken together, for clinical application, a RT-PCR procedure for PSA for detection of circulating prostate cells should ideally define the sensitivity of the assay under clinical conditions, independent of calibration by LNCaP cells, and correct for (inconsistent) test results associated with false positives arising from leukocytes (iPSA mRNA) and possibly PSA mRNA from circulating nonmalignant prostate cells or with false negatives attributable to random detection of low-abundance PSA mRNA of PCa cell origin.

In the present study, we showed that multifold testing and appropriate statistical handling enable the quantification of intraassay variation and the determination of the number of required test replicates and number of positive tests from among these replicates to conclude true posi-

tivity at a diagnostic specificity of ≥98%. In addition, the proposed procedure provides an alternative indicator of the sensitivity of a RT-PCR assay for PSA mRNA as the best estimate of the mean (λ) of the expected Poisson frequency distribution of positive PSA PCR tests among replicates. Such a "λ value" characterization can be applied to compare the performance of (different) RT-PCR protocols for PSA or with other, similarly characterized RT-PCR assays, such as the RT-PCR assay for prostate-specific membrane antigen. It should be noted, however, that this approach was developed by analyzing the results of four institutions as one cohort of data (or, strictly speaking, in the context of an interlaboratory setting) and that intraassay variation may vary from laboratory to laboratory. Therefore, clinical application of the outlined approach requires "local" characterization (λ value) of even technically identical RT-PCR protocols.

Adjustment of the limit of detection to eliminate interference by iPSA mRNA transcripts has frequently been suggested but addressed only to a limited extent *(9, 10, 14)*, most likely because of the absence of a reliable calibrator for the sensitivity of the assay. A numeric approach such as the one outlined in the present study can be of value in this respect. Confirming the observations of others *(9, 10, 14)*, our data showed that, compared with the high-sensitivity (35 × 2 PCR cycles; λ = 1.0) assay, the relative, less sensitive (25 × 2 PCR cycles; λ = 0.2) PCR assay was affected to a lower degree by iPSA mRNA transcripts without a considerable reduction in the detection of true positives. Assuming the establishment of a well-characterized assay (number of necessary test replicates, statistics, and other conditions), the presented data showed, however, that clinically relevant data could be obtained without the extensive procedure of adjusting the assay sensitivity. On the other hand, the development of a less sensitive RT-PCR assay for PSA mRNA without loss of diagnostic sensitivity may be cost-effective because the number of necessary PCR test replicates is reciprocally related to the sensitivity of the assay.

Recently, new RT-PCR procedures for PSA mRNA have been designed, such as quantitative assays with PSA mRNA(-like) internal or external standards *(18, 19, 22)*. These advanced RT-PCR procedures for PSA intend to control tube-to-tube variation and to determine the clinically significant minimal number of PSA mRNA transcripts in PCa patients. The clinical impact of these novel RT-PCR procedures for PSA in molecular staging remains to be established. The benefits of more complicated and, currently not generally available, quantitative RT-PCR assays for PSA are disputable. At present, it is not possible to quantify the number of circulating prostate cells from quantified PSA mRNA because the transcription rate (i.e., the amount of target RNA) varies among tumor cells. For this reason, no additional, clinically relevant information can be expected from a quantitative RT-PCR assay for PSA compared with a well-characterized qualitative (nested) RT-PCR procedure.

A serious disadvantage of any RT-PCR procedure for PSA mRNA, including the one presented here, is the inability to distinguish between healthy and malignant cells of prostate origin. Additional shedding of nonmalignant prostate cells parallel to intravasion of tumor cells and survival over a considerable period of time of those cells expressing PSA mRNA transcripts cannot be excluded a priori. Furthermore, if the expression of PSA mRNA in circulating prostate (cancer) cells of patients depends on the degree of differentiation, progression to hormone-refractory PCa, or androgen ablation treatment, the RT-PCR assay for PSA may generate clinically false results *(4, 5, 23)*. Consequently, to solve these biologically important and clinically relevant aspects, research efforts should focus on enrichment of epithelial prostate (cancer) cells from the circulation, followed by cell quantification *(24)*. This, combined with a quantitative RT-PCR assay for PSA and PCa cell-specific DNA probes and antibodies, can lead to the generation of major, basic, and clinically relevant information.

In conclusion, numeric assessment of a nested RT-PCR assay for PSA mRNA provides a sensitive and specific methodology for initial detection of the presence of blood-borne PCa cells. As long as sensitive and reliable methods for direct quantification and characterization of circulating prostate (cancer) cells are not routinely available, the general application of well-defined RT-PCR assays for tissue-specific targets may enable reliable comparisons between clinical studies and contribute to a definite introduction of RT-PCR assays for PSA mRNA in the clinical practice.

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