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Thyroid Fine-Needle Aspiration Samples Inadequate for Reverse Transcriptase PCR

Analysis

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Original Article

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Running title: Thyroid FNA Samples Inadequate for RT-PCR

Condensed abstract:

Inadequate samples for RT-PCR analysis were studied on 350 thyroid fine-needle aspiration samples. Proportion of inadequate samples for RT-PCR was higher in samples from leftover material in the needle (21.7%) than in samples from separate puncture (13.1%) ($p=0.049$) and

no correlation between the adequacy of samples for RT-PCR and the largest diameter of the nodule was found.

Abstract:

Background. Analysis of different tumor markers by reverse transcriptase polymerase chain reaction (RT-PCR) in fine-needle aspiration samples of thyroid nodules has been studied with the aim to improve the accuracy of preoperative diagnosis of thyroid lesions. The aim of this study was to investigate inadequate thyroid fine-needle aspiration samples for RT-PCR analysis and to determine if there is a correlation between their proportion and method of sampling or diameter of nodules.

Methods. A total of 350 fine-needle aspiration samples from patients with thyroid nodules were analyzed. After the aspirate was smeared for conventional cytology the leftover material in the needle was used for RT-PCR analysis in one group of 175 patients. In another group of 175 patients separate puncture was performed to obtain material only for RT-PCR analysis.

Samples were considered adequate for RT-PCR analysis if the expression of both glyceraldehyde-3-phosphate dehydrogenase and thyroglobulin was found by RT-PCR.

Results. In total, 61 (17.4%) inadequate samples for RT-PCR were detected. All 12 samples that were inadequate for cytological diagnosis were also inadequate for RT-PCR analysis.

Proportion of inadequate samples for RT-PCR was significantly higher in samples from leftover material in the needle (21.7%) than in samples from separate puncture (13.1%) ($p=0.049$). No statistically significant correlation between the adequacy of samples for RT-PCR and the largest diameter of the nodule was found.

Conclusion. Proportion of inadequate samples for RT-PCR was higher in samples from leftover material in the needle than in samples from separate puncture.

Key words:

biopsy, fine-needle; glyceraldehyde-3-phosphate dehydrogenase (phosphorylating); reverse transcriptase polymerase chain reaction; thyroglobulin; thyroid neoplasms; thyroid nodules

Introduction

Palpable thyroid nodules are very common with the prevalence of 4% to 7% among the adults in North America (1,2). But only 5% to 10% of palpable thyroid nodules are malignant (3). Cytological analysis of material obtained by fine-needle aspiration biopsy (FNAB) is quick, cost-effective, highly accurate, and minimally invasive method used for distinguishing benign from malignant thyroid nodules preoperatively (4,5). Although this method enables accurate identification of most thyroid lesions, it is difficult to distinguish benign (hyperplastic nodules, follicular adenoma, Hurthle cell adenoma) from malignant (follicular carcinoma, Hurthle cell carcinoma, and follicular variants of papillary carcinoma) follicular thyroid lesions by FNAB cytology (4,6,7). Also, in a proportion of patients definitive diagnosis can not be made by FNAB cytology due to inadequate samples (8). Therefore, expression of different molecular markers in FNAB samples has been analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) with the aim to improve preoperative diagnostic accuracy of thyroid lesions, particularly in patients with follicular thyroid lesions or inadequate FNAB samples. The expectations from RT-PCR analysis of thyroid FNAB samples are in the first place to help establish preoperative diagnosis regarding malignancy in cytologically indeterminate samples, but also in a second place to reduce the number of inadequate samples, based on the hypothesis that smaller number of cells is needed for RT-PCR than for cytological analysis. Some of the markers analyzed have shown promising results and many new ones are emerging as a result of microarray analysis of thyroid lesions (9-16).

Still, in most of the studies, inadequate FNAB samples for RT-PCR analysis have not been investigated, and in a proportion of studies even their number has not been explicitly reported. In other studies in which the number of thyroid FNAB samples inadequate for RT-PCR

analysis has been explicitly reported, it varied significantly. In all of these studies the number of FNAB samples investigated was smaller than in our study.

The aim of this study was to investigate inadequate thyroid FNAB samples for RT-PCR analysis and to determine if there is a correlation between their proportion and the method of sampling (needle leftover material versus separate puncture) or the diameter of nodules.

Takano et al. were first to show in 1997 that sufficient material for RT-PCR analysis can be obtained from leftover material in the needle (17). They have shown on one sample that RNA prepared from leftover cells could produce the RT-PCR products similar to those using RNA from a whole aspirate (17). Since then leftover material was analyzed by RT-PCR in most studies on preoperative FNA samples. The major advantage of using leftover material for RT-PCR analysis instead of performing separate puncture is that both cytological analysis and RT-PCR analysis can be performed without additional invasion to the patient. However, these two approaches (leftover material and separate puncture) have not been compared so far on more than one FNAB sample.

The adequacy of samples for RT-PCR analysis was evaluated by detection of two transcripts: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and thyroglobulin. GAPDH was analyzed as a housekeeping gene control marker to confirm the integrity of isolated RNA and efficiency of reverse transcription reaction. Thyroglobulin was analyzed as control marker for the presence of thyroid follicular cells due to its strong and specific expression in thyroid cells of follicular origin. In other studies, GAPDH, β -actin, and PGK-1 were analyzed as housekeeping gene control markers. Although GAPDH is widely used as housekeeping gene control, it has been shown that the expression level of GAPDH, and also that of other housekeeping gene control markers, can vary in response to different factors including malignancy of the cells (18,19).

As control marker for presence of thyroid follicular cells, thyroglobulin was analyzed in all but one study in which thyroid-stimulating hormone receptor (TSH-R) was analyzed (20). All 20 FNAB samples were positive for TSH-R in that study. Thyroglobulin is analyzed because GAPDH or any other housekeeping gene would also reflect the presence of only blood cells in the sample, in which case the sample would be inadequate for RT-PCR analysis of markers for malignancy. Furthermore, it has been shown that lymphocytes can be responsible for false positive findings when hTERT was analyzed as marker for malignancy, and macrophages can be responsible for false positive findings when galectin-3 was analyzed as marker for malignancy (21,22). In those cases it would be possible that the sample is positive for both housekeeping gene control marker and the marker for malignancy, although it is inadequate for RT-PCR analysis. This situation can be avoided if the marker for the presence of thyroid follicular cells, as thyroglobulin, is analyzed. Of course, false positive adequate samples can not be avoided that way.

In a few studies only thyroglobulin and no housekeeping genes were analyzed (23,17,24). This is reasonable because positive finding of thyroglobulin expression is sufficient to pronounce thyroid FNAB sample adequate for RT-PCR analysis. In any event, one should be cautious in interpreting negative findings of thyroglobulin expression. High variability in thyroglobulin expression in thyroid tissues (normal, benign, and malignant) ranging from 0.05 – 367 fmol/μg RNA has been shown (25). Also, decreased expression of thyroglobulin compared to normal thyroid tissue has been shown in follicular carcinomas, follicular adenomas, and papillary carcinomas (26). In one study archival Diff-Quik stained slides were analyzed for the expression of thyroglobulin as marker of follicular thyroid cells and hTERT as marker of malignancy by RT-PCR, and 5 out of 58 samples were positive for hTERT and negative for thyroglobulin. Because thyroid follicular cells were confirmed on corresponding slides by cytopathology, authors pronounced these samples adequate for RT-PCR, and

explained the lack of detection of thyroglobulin gene expression by the fact that a subset of thyroid malignancies does not express thyroglobulin gene (27). If RT-PCR is used to analyse thyroid lesions of non-follicular cell origin (e.g. medullary carcinoma), then thyroglobulin can not be analyzed as control marker to confirm adequacy of material.

Materials and Methods

Patients and samples

Ultrasound-guided FNAB was performed as a part of the standard diagnostic protocol for patients with thyroid nodules in Department of Oncology and Nuclear Medicine, University Hospital “Sestre milosrdnice”, Zagreb, Croatia. One to three punctures per nodule, depending on the size of the nodule, were performed by a cytopathologist using 25-gauge needle. The maximal diameter of the aspirated nodule was measured by ultrasonography. Samples from lesions with a satisfactory cytological diagnosis other than that of follicular cell origin (intrathyroid parathyroid adenoma and medullary carcinoma) were excluded from the study. Samples in which no groups of well preserved follicular cells were present were considered cytologically inadequate. All cytologically adequate samples were semiquantitatively assessed for cellularity and each one was scored as 1 (few follicular cells), 2 (moderate number of follicular cells), and 3 (many follicular cells). A total of 350 thyroid nodule samples from patients (299 female, 51 male) aged 18-81 years (median age 52.8 years) collected between January 2003 and June 2007 were included in the study. Informed consent according to the World Medical Association Declaration of Helsinki was obtained from all the patients before their inclusion in the study and the study was performed in conformance with the Declaration of Helsinki ethical guidelines (28). Patients were randomized to two groups. After the aspirate was smeared for conventional cytology (May-Gruenwald-Giemsa staining) the leftover material in the needle was used for RT-PCR analysis in one group (175 patients). In another 175 patients separate puncture of the same nodule was performed to obtain material only for RT-PCR analysis. Samples were considered adequate for RT-PCR analysis if the expression of both GAPDH and thyroglobulin was found by RT-PCR.

RNA Isolation and Reverse Transcription

Total cellular RNA was isolated from aspirates using the TriPure Isolation Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's instructions (29). For reverse transcription (RT), 5 μ L of RNA was mixed with 0.5 μ g Oligo d(T)18 primer (New England Biolabs, Beverly, MA, USA) and incubated for 4 minutes at 70 °C. After that, other components were added to this mixture in the final concentrations of 1 x RT buffer (New England Biolabs, Beverly, USA), 0.5 mmol/L of each dNTPs (Sigma, Saint Louis, MO, USA), 1 U/ μ L RNase inhibitor (Roche, Mannheim, Germany) and 1.25 U/ μ L M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA) in a total volume of 20 μ L. The mixture was first incubated for 60 minutes at 42 °C, then for final 5 minutes at 94 °C, and stored at -20 °C.

PCR Reaction

Expressions of thyroglobulin and GAPDH were analyzed by PCR. For the PCR analysis 2 μ L of RT mixture were added to the PCR reaction mixture containing final concentrations of 1 x Taq buffer with Mg^{2+} (Eppendorf, Hamburg, Germany), 0.2 mmol/L of each dNTPs (Sigma, Saint Louis, MO, USA), 0.2 μ mol/L sense primer, 0.2 μ mol/L antisense primer, and 0.02 U/ μ L Taq DNA polymerase (Eppendorf, Hamburg, Germany) in a total volume of 25 μ L. PCR reaction conditions for analysis of GAPDH expression were: one cycle of 5 minutes at 95 °C followed by 30 cycles each consisting of one minute at 95 °C, one minute at 55 °C, and 45 seconds at 72 °C, followed with a final 10-minute extension at 72 °C. PCR reaction conditions for analysis of thyroglobulin expression were: one cycle of 5 minutes at 95 °C followed by 32 cycles each consisting of one minute at 95 °C, 30 seconds at 60 °C, and 45 seconds at 72 °C, followed with a final 10-minute extension at 72 °C.

The previously described oligonucleotide primers were used for GAPDH and thyroglobulin cDNA amplification (30,31) (Table 1).

All PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide and directly visualized under UV light at 302 nm. DNA molecular weight markers VIII or IX (Roche, Manheim, Germany) were included in all gels. A sample was regarded as positive if a band of expected size (623 bp for GAPDH, and 529 bp for thyroglobulin) was present.

Statistical analysis

Statistical significance of association between the distribution of the values of RT-PCR adequacy of samples on one side and method of sampling and cellularity on the other side was determined by Chi-square test. Statistical significance of association between the distribution of the values of RT-PCR adequacy of samples and diameter of nodules was determined by Mann-Whitney U test. The analysis was performed using software STATISTICA, version 5.5 (StatSoft Inc., Tulsa, OK, USA) and a P value of ≤ 0.05 was considered statistically significant.

Results

A total of 61 (17.4%) inadequate samples for RT-PCR were detected in 350 samples analyzed (Table 2). Of these 61 inadequate samples, 40 samples were negative for both GAPDH and thyroglobulin, and 21 samples were positive for GAPDH and negative for thyroglobulin.

The proportion of inadequate samples for RT-PCR was significantly higher in samples from leftover material in the needle than from samples obtained with a separate puncture (Chi-square test, $p=0.049$) (Table 2).

The largest diameter of the punctured nodule was measured by ultrasonography for 318 (90.9%) samples with a mean of 21.5 mm (range: 4-94 mm). Among the group of 267 patients with adequate samples for RT-PCR, the mean largest nodule diameter was 21.7 mm (range: 4-94 mm). In the group of 51 patients with inadequate samples for RT-PCR, the mean largest nodule diameter was 20.7 mm (range: 6-80 mm). No statistically significant correlation was found between the adequacy of samples for RT-PCR and the largest diameter of the nodule (Mann Whitney U Test, $p=0.91$).

Twelve of the 350 samples (3.4%) were inadequate for cytological diagnosis and all 12 samples were also inadequate for RT-PCR analysis (Table 3). These samples consisted of blood or colloid only with no follicular cells present.

Forty-nine of the 350 samples (14.0%) were inadequate for RT-PCR analysis but adequate for cytological diagnosis. Of these 49 samples 19 were obtained by separate puncture and 30 were obtained from leftover material in the needle. Thirty-two of these 49 samples were negative for both GAPDH and thyroglobulin and 17 were positive for GAPDH and negative for thyroglobulin.

Among 167 cytologically adequate samples in which leftover material in the needle was analyzed by RT-PCR statistically significant association between the adequacy of samples for RT-PCR and their cellularity was found (Chi-square test, $p<0.001$) (Table 4). Among 171

cytologically adequate samples obtained with a separate puncture no association was found between the adequacy of samples for RT-PCR and cellularity of corresponding sample analyzed by cytopathology (Chi-square test, $p=0.372$).

Discussion

In this study, 61 (17.4%) of 350 thyroid FNAB samples were inadequate for RT-PCR analysis which is in the range of the proportions obtained in other studies all performed on smaller sample. In study by Niedziela et al, 7 out of 30 (23.3%) in vivo FNAB samples were negative for thyroglobulin expression (32). In that study, as in our study, GAPDH was also investigated as control marker, but the number of GAPDH negative samples has not been reported. In a study by Cheung et al, 2 out of 75 (2.7%) in vivo FNAB samples were negative for thyroglobulin expression and all samples were positive for PGK-1 expression (33). In another study in which PGK-1 was analyzed as housekeeping gene control marker, only 3 out of 8 intraoperatively obtained FNAB samples were positive for PGK-1 expression (34). In the study by Domingues et al, in which GAPDH and thyroglobulin were analyzed as control markers, 4 out of 63 (6.3%) in vivo FNAB samples were inadequate for RT-PCR analysis (35). In a study by Giannini et al, all 65 samples analyzed were positive for both GAPDH and thyroglobulin (36). In that study ex vivo FNAB samples were analyzed. It is much easier to obtain abundant and adequate material by ex vivo than in vivo fine-needle aspiration, so the proportion of inadequate FNAB samples for RT-PCR analysis should be lower in ex vivo FNAB samples. In one study by Takano et al, all 72 ex vivo FNAB samples were positive, while 9 out of 177 (5.1 %) in vivo FNAB samples were negative for thyroglobulin expression (23). In another study by the same group no inadequate samples were found among 35 ex vivo and 3 in vivo FNAB samples (37). In the study by Karger et al, adequate material for real-time RT-PCR analysis was obtained in more than 90% of 244 in vivo FNAB samples analyzed (38). In one study ~10% of all in vivo FNAB samples showed an extremely low copy number of oncofetal fibronectin (malignancy marker) and thyroglobulin mRNA and were not used for further real-time RT-PCR analysis because authors concluded that these samples were likely to contain many contaminating blood cells rather than thyroid tumor cells

(26). In a study by Siddiqui et al, archival Diff-Quik stained slides were analyzed by RT-PCR and 10 out of 58 (17.2%) samples were considered inadequate for RT-PCR by the authors because no expression of any marker analyzed, including β -actin and thyroglobulin, was found (27). In one study in vivo FNA samples from lymph nodes were analysed by RT-PCR for the presence of thyroid cancer metastases, and 5 out of 46 (10.9%) samples were negative for GAPDH expression (39). In several other studies, all performed on a small sample (3 to 21 FNAs), no inadequate samples for RT-PCR were found (20,40-44). In some studies the exact number of inadequate samples for RT-PCR analysis has not been explicitly reported (17,21,24,45-49).

In our study samples were analyzed by conventional RT-PCR where PCR products were detected by electrophoresis on agarose gels. The same method was used in the majority of other studies in which thyroid FNAB samples were analyzed by RT-PCR. The analysis of samples by quantitative real-time RT-PCR should enable higher sensitivity for the detection of markers and consequently smaller proportion of samples inadequate for RT-PCR analysis. This approach was studied in only few studies and 0 to 10% of samples inadequate for real-time RT-PCR analysis were reported (14,16,26,38,50).

All samples that were negative for GAPDH were also negative for thyroglobulin in our study. Also in other studies no samples negative for housekeeping gene control markers and positive for thyroid follicular cell-specific markers were reported. Still, it is possible if only few cells or theoretically a single cell is present in the FNAB sample that the expression level of housekeeping gene is below and the expression level of thyroid follicular cell-specific gene is above the threshold of the method.

In our study all 12 samples that were inadequate for cytological diagnosis were also inadequate for RT-PCR analysis. In several other studies a proportion of cytologically inadequate and RT-PCR adequate samples was reported (23,33,38,39,40,42). However, the

proportion of cytologically inadequate samples in all of these studies was significantly higher than in our study (23,33,38,39,40). In one study 2 out of 75 samples were inadequate for RT-PCR analysis, and among remaining 73 samples adequate for RT-PCR analysis 22 (30.1%) were inadequate for cytological analysis (33). Very high proportion of cytologically inadequate samples might be explained by ThinPrep methodology used for cytological evaluation in that study. In one study FNAB samples from lymph nodes were analyzed by RT-PCR for the presence of thyroid cancer metastases, and 5 out of 46 samples (10.9%) were inadequate for RT-PCR, while 13 out of 46 samples (28.2%) were inadequate for cytological diagnosis (39). Authors explained a high proportion of cytologically inadequate samples by the fact that only small (diameter <1.5cm) lymph nodes were analyzed in their study. In one study adequate material for real-time RT-PCR analysis was obtained in more than 90% of 244 FNAB samples analyzed, while 43 out of 244 samples (17.6%) were inadequate for cytological diagnosis (38). However, in that study samples obtained from five different institutions were analyzed and the proportion of cytologically inadequate samples varied from 5% to 20% per institution, which might indicate that the rate of cytologically inadequate samples depends on the experience and skills of person that performs FNAB (38). Takano et al reported a case of a patient in which two FNAB samples yielded cytologically insufficient material because of poor fixation and scant number of tumor cells, while the same FNAB samples were adequate for RT-PCR analysis (42). However, the aspiration in that case was performed without using ultrasound which could explain obtaining cytologically insufficient material. Our results suggest that if ultrasound-guided FNA is performed by an experienced physician (in our institution it is performed by cytopathologist) then it is unlikely that RT-PCR analysis would significantly decrease the number of inadequate samples. Accordingly, we suggest that in the case of inadequate material for cytological analysis, repeated FNAB

and cytological analysis should be performed because it significantly reduces the proportion of cytologically inadequate samples (51).

In our study 49 of the 350 samples (14.0%) were inadequate for RT-PCR analysis but adequate for cytological diagnosis. Nineteen of these samples were obtained by separate puncture. It is possible that no follicular cells were present in the material obtained for RT-PCR analysis while they were present in the material obtained for cytological analysis. In the remaining 30 of these samples leftover material in the needle was analyzed by RT-PCR. The cellularity in these samples was statistically significantly lower than in the samples from leftover material that were adequate both cytologically and for RT-PCR. Low cellularity could explain inadequacy of these samples for RT-PCR analysis. Decrease in the number of follicular cells present in the sample increases the chance that the number of follicular cells present in the leftover material will be below the threshold for detection of RT-PCR.

Mitteldorf et al have also shown the correlation between the cellularity of FNAB samples and their adequacy for RT-PCR analysis (52). Still, among 30 samples in which leftover material was analyzed for RT-PCR and were inadequate for RT-PCR analysis and adequate for cytological diagnosis there were 8 samples with many follicular cells present. Takano et al have offered possible explanation for such results suggesting that sometimes when large volume of blood or cystic fluid is aspirated, the majority of follicular cells in the aspirate can be washed out onto the slide glass with no or only few follicular cells remaining in the leftover material (23). We did not keep the record of the amount of blood or cystic fluid in our aspirates so we can not confirm that this was the case in our samples. Still, we can not offer any other explanation for these results since our RT-PCR inadequate samples were in no other quality observed significantly different from our RT-PCR adequate samples. We are planning to investigate the influence of blood or cystic fluid in the aspirates on their adequacy for RT-PCR analysis on samples we are going to collect in the future.

Unlike in the samples from leftover material, in samples obtained with a separate puncture no association was found between the adequacy of samples for RT-PCR and cellularity. This result is expected because the cellularity was assessed in samples obtained for cytological analysis, which is not necessarily the same as the cellularity in corresponding samples that were analyzed by RT-PCR.

In our study, the proportion of inadequate samples for RT-PCR was higher in samples from leftover material in the needle (21.7%) than in samples from separate puncture (13.1%) ($p=0.049$). This could be because more cells are present and consequently more RNA can be extracted from separate puncture. Takano et al. could extract less than 50 ng of total RNA from leftover material in the needle, while Lubitz et al. could extract 137 ng to 5.8 μg (mean, 1.7 μg) of total RNA from separate puncture and Kebebew et al. could extract 406 ng/ μL to 914 ng/ μL of total RNA (in 30-60 μL volume), also from separate puncture (14,15,17).

Although we have shown lower proportion of inadequate samples in material obtained by separate puncture, our results do not justify performing a separate puncture instead of using leftover material for RT-PCR analysis in all patients. The increase in the proportion of adequate samples would be only 8.6% in our sample and all 100% of patients would be subjected to additional invasion. Only in those cases in which inadequate material for RT-PCR and adequate material for cytological analysis was obtained, should an additional puncture for RT-PCR analysis be considered.

In conclusion, in our study the proportion of inadequate samples for RT-PCR was higher in samples from leftover material in the needle than in samples from separate puncture and no correlation between the adequacy of samples for RT-PCR and the largest diameter of the nodule was found.

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Table 1. Sequences of oligonucleotide primers used for detection of target genes by RT-PCR

Target gene	Primer	Sequence	Product size (bp)
GAPDH	GAPDH1 (sense)	5'-AAC GGA TTT GGT CGT ATT GGG C-3'	623
	GAPDH2 (antisense)	5'-AGG GAT GAT GTT CGT GAG AGC C-3'	
Thyroglobulin	TG1 (sense)	5'-GCC TCC ATC TGC TGG GTG TC-3'	529
	TG2 (antisense)	5'-CTC CCT CCG CAG AAC ACT GGG GT-3'	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2. Distribution of 350 thyroid fine-needle aspiration biopsy samples regarding their adequacy for RT-PCR and method of sampling.

Method of sampling	Samples		
	Inadequate material for RT-PCR /number (%)/	Adequate material for RT-PCR /number (%)/	Total /number/
Separate puncture	23 (13.1%)	152 (86.9%)	175
Needle leftover	38 (21.7%)	137 (78.3%)	175
Material			
Total	61 (17.4%)	289 (82.6%)	350

Chi-square test, p=0.049

Table 3. Distribution of 12 cytologically inadequate thyroid fine-needle aspiration biopsy samples regarding expression of GAPDH and thyroglobulin detected by RT-PCR and method of sampling.

Method of sampling	Samples		
	GAPDH negative, thyroglobulin negative (number)	GAPDH positive, thyroglobulin negative (number)	Total (number)
Separate puncture	2	2	4
Needle leftover material	6	2	8
Total	8	4	12

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 4. Distribution of 167 cytologically adequate thyroid fine-needle aspiration biopsy samples in which leftover material in the needle was analyzed by RT-PCR regarding their adequacy for RT-PCR and cellularity.

	Samples			Total
	Cellularity score	Cellularity score	Cellularity score	
Adequacy for RT-PCR	1 /number (%)/	2 /number (%)/	3 /number (%)/	/number/
Adequate	3 (2.2%)	57 (41.6%)	77 (56.2%)	137
Inadequate	5 (16.7%)	17 (56.7%)	8 (26.7%)	30
Total	8 (4.8%)	74 (44.3%)	85 (50.9%)	167

cellularity score 1 – few follicular cells, 2 – moderate number of follicular cells, 3 – many follicular cells

Chi-square test, $p < 0.001$