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Original Scientific Paper

CLINICAL APPLICATION OF RT-PCR IN TUBERCULOSIS DNA DETECTION COMBINED WITH TB-IGRA IN THE DIAGNOSIS OF SPUTUM SMEAR-NEGATIVE PULMONARY TUBERCULOSIS

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SUMMARY – The aim was to investigate detection of pulmonary alveolar lavage fluid tuberculosis DNA by real-time fluorescent polymerase chain reaction (RT-PCR) combined with clinical application of the sputum smear-negative pulmonary tuberculosis diagnosis with TB interferon-γ release assay (TB-IGRA). From October 2014 to October 2015, 632 outpatients and inpatients treated in our hospital were randomly selected, of which 459 patients as the research group managed with RT-PCR detection combined with TB-IGRA and 173 patients as the control group undergoing electronic bronchoscopy alveolar lavage fluid detection, with detection results statistically evaluated. The positive rate in the research group was 96.51%, i.e. significantly higher than that in the control group (66.47%), yielding a statistically significant difference (χ^2=109.68, p=0.00). The true positive rate was 97.7% in the research group and 67.92% in the control group; the true positive rate was significantly higher in the research group patients as compared with the control group, yielding a statistically significant difference (χ^2=112.04, p=0.00). The sensitivity and specificity, as well as Youden index were significantly higher in the research group as compared with the control group. In conclusion, TB DNA detection by RT-PCR combined with TB-IGRA is a very good method of diagnosing tuberculosis, and it can be implemented in clinical diagnosis of pulmonary tuberculosis.

Key words: *RT-PCR; TB-IGRA; Alveolar lavage fluid; Diagnosis*

Introduction

At present, the incidence and mortality rate of tuberculosis (TB) are very high. Control of TB epidemic is not very effective, and one of the important reasons for this is low efficiency of its diagnosis[1]. Over the past century, tuberculin skin test (TST) was commonly used for TB screening and has become the most popular method of TB diagnosis, but this method cannot distinguish historical TB infection from new infection, and the existence of non-tuberculous bacillus and Bacillus Calmette Guerin (BCG) can easily lead to false-positive result due to cross immunity, while in people with immune suppression, it is very often prone to be false-negative[2]. All of these disadvantages limit clinical application of TST[3,4]. Currently, the gold standard of TB diagnosis should include microbiological TB culture positivity, TB polymerase chain reaction (PCR), histopathology and immunology results. However, it is quite

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complicated and takes some time, so it is particularly important to develop a method with higher efficiency in the diagnosis of TB.

Real-time fluorescent polymerase chain reaction (RT-PCR) can specifically detect certain DNA sequences. In this method, DNA is amplified and the amplified products are analyzed by detection of fluorescence in a free R group of 5' terminal fluorescent probe, i.e., if the target gene exists in the PCR system, target nucleic acid fragments would be amplified through PCR, and the fluorescent probe will be displayed by base pairing[5-7]. Usually, the more copies are detected in bronchoalveolar lavage (BAL) by the RT-PCR method, the higher is the positive rate[8]. The interferon-gamma release assay (TB-IGRA) is a technique for TB diagnosis based on the principle that T lymphocytes in TB patients would secrete interferon-γ under specific antigen stimulation. TB-IGRA reaction is positively correlated to the number of *Mycobacterium tuberculosis*[9-11]. The main advantage of this method is that it can eliminate the interference caused by non-tuberculosis causes in patients and can be used for epidemiologic investigations. At present, TB-IGRA has been applied in TB diagnosis both in China and other countries[12-14]. Detection of alveolar lavage fluid by electron bronchoscopy is also a clinical diagnostic method of detecting TB, but clinical research showed the positive rate to be low, whereas other studies report that the positive rate could reach 80.9%. Its diagnosis rate can be improved by pathologic biopsy, culture and brushing, thus avoiding missed diagnosis and misdiagnosis[15-18].

This study aimed to explore the value of RT-PCR combined with TB-IGRA in TB diagnosis, in order to find out an effective diagnostic method of TB.

Materials and Methods

Patients

A total of 632 TB patients, outpatients and inpatients treated in our hospital from October 2014 to October 2015, were randomly selected and included in the study. All these patients were diagnosed with TB according to the pulmonary tuberculosis diagnostic criteria of the Ministry of Health of the People's Republic of China[19], and all underwent BAL. The patients were divided into the research group and control group according to the method of TB diagnosis. The research group patients underwent detection in BAL fluid by electronic TB DNA combined with TB-IGRA. This group included 459 patients, 242 male and 217 female, age range 17-70 years, mean age 37.50±15.20 years. Control group patients underwent electronic detection in BAL. This group included 173 patients, 101 male and 72 female, age range 16-75 years, mean age 36.9±14.75 years. As a result, 435 patients were diagnosed with TB infection in the research group, while 24 patients were uninfected. In the control group, 159 patients were diagnosed with TB infection, while 14 patients were without infection. Differences in age, gender and other general data between the two groups were not statistically significant.

The study was approved by the institutional Ethics Committee and informed consent was signed by the patients participating in the study.

Methods

RT-PCR method: Patient bronchus was examined by bronchoscopy. Patients with abnormalities underwent BAL, or BAL was performed in the abnormal area found on computed tomography. In each patient, 5 mL of BAL fluid was collected under aseptic conditions. BAL fluid was detected by RT-PCR according to the instructions of the TB-DNA detection kit.

TB-IGRA: Interferon-γ detection kits were used (Beijing Wantai Biological Pharmaceutical Co., Ltd., Product Registration Number: State Food and Drug Administration device (Approved) number 2012 No. 3400557). One mL of heparinized peripheral blood was collected from each patient and packed in a test culture tube, control culture tube, and negative control culture tube, and cultured at 37 ℃ for 24 hours, centrifuged for 10 minutes, and the supernatants were detected by enzyme-linked immunosorbent assay (ELISA). Absorbance (A) was determined by a Bio-Rad 680 Microplate Reader. The interferon-γ content value of the negative control culture tube (N), interferon-γ content value of the test culture tube (T), interferon-γ content value of the positive control culture tube (P) (unit: pg/mL): when N≤400, P-N=any value, T-N≥14 and ≥N/4, the result was determined as positive; when N≤400, P-N≥20, T-N<14, the result was determined as negative; when N≤400, P-N≥20, T-N≥14 but <N/4, the result was determined as negative; when N≤400, P-N<20, T-N<14, the result was determined as uncertain; when N≤400, P-N<20, T-N≥14 but

<N/4, the result was determined as uncertain; when N≥400, P-N=any value, T-N=any value, the result was determined as uncertain. Final results were expressed in three ways, i.e., as positive, negative, and uncertain.

Electronic bronchoscopy detection method: fasting for 6 hours before detection, 5-6 mL of 2% lidocaine was used for inhalation anesthesia, detection with electronic bronchoscopy (Olympus CV-150, Japan); biopsy specimens of patients with abnormal bronchoscopy results were sent for pathologic examination; and brushing detection in BAL fluid, each used to produce three smear samples to send for *Mycobacterium tuberculosis* detection. BAL was used in patients without abnormalities.

Statistical analysis

In this study, SPSS 20.0 statistical software was used for statistical analysis. Numerical data were expressed as n (%), and statistical analysis of numerical data was performed using the χ^2-test. The level of statistical significance of differences was set at $p<0.05$.

Results

Positive rate comparison

In the research group, the rate of positive findings was 96.51%, i.e., significantly higher than that in the control group (66.47%), yielding a statistically significant difference ($\chi^2=109.68$, $p=0.00$) (Table 1).

Sensitivity detection

The true positive rate was 97.7% in the research group and 67.92% in the control group. The true positive rate was significantly higher in the research group patients as compared with the control group, yielding a statistically significant difference ($\chi^2=112.04$, $p=0.00$) (Table 2).

Sensitivity and specificity comparison

Through comparison, we found that the sensitivity and specificity, as well as Youden index were significantly higher in the research group as compared with the control group (Table 3).

Table 1. Comparison of positive rate between two groups of patients

Group	Positive (n)	Negative (n)	Positive rate
Research group (N=459)	443	16	96.51%
Control group (N=173)	115	58	66.47%
χ^2 value			109.68
p value			10^{-7}

Table 2. Sensitivity of two methods

Group	Actual	Positive, n (%)	Negative, n (%)
Research group (N=459)	TB positive (n=435)	425 (97.70)	10 (2.04)
	TB negative (n=24)	4 (16.67)	20 (83.33)
Control group (N=173)	TB positive (n=159)	108 (67.92)	51 (32.08)
	TB negative (n=14)	7 (50.00)	7 (50.00)

Actual = cases confirmed as TB according to the pulmonary tuberculosis diagnostic criteria of the Ministry of Health of the People's Republic of China; Positive/Negative = cases detected as TB positive/negative by either electronic bronchoscopy alveolar lavage fluid TB DNA combined with TB-IGRA, or electronic bronchoscopy alveolar lavage only

Table 3. Comparison of sensitivity, specificity, and other indexes (%)

Group	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden index
Research group (N=459)	97.75	87.50	99.54	41.67	85.25
Control group (N=173)	75.71	66.67	95.78	78.46	42.38

PPV = positive predictive value; NPV = negative predictive value; Youden index: true positive rate-false positive rate (accuracy)

Discussion

The gold standard of TB diagnosis is complicated. Therefore, we tried to find out a more efficient method with high accuracy and sensitivity. RT-PCR can specifically detect TB DNA, and the more copies are detected in BAL by the RT-PCR method, the higher is the positive rate[8]. TB-IGRA is a technique with high sensitivity and high specificity[9-11]. The main advantage of this method is that it can eliminate the interference caused by non-tuberculosis causes in patients and can be used for epidemiologic investigations. In this study, RT-PCR was combined with TB-IGRA to see whether this combination could reduce the rates of missed diagnosis and misdiagnosis.

Our results showed that positive rate of the combined detection method was 96.51%, sensitivity 97.75%, specificity 87.50%, and Youden index 82.25. Thus, it is clear that the positive rate, sensitivity and specificity were greatly improved by the combined RT-PCR and TB-IGRA detection method, while showing obvious advantages compared with the BAL fluid detection by using the electronic bronchoscopy method. However, clinical studies have shown that in immunocompromised patients, the positive rate of TB-IGRA is not high due to T lymphocyte reduction[20]. Our study did not explore these problems, thus more data are needed for further analysis. On the other hand, there was no experimental comparison between bacterial positive and bacterial negative pulmonary TB, but research showed that there was no statistical difference in the interferon-γ secretion between them.

In RT-PCR, DNA is amplified, and the number of amplified products is analyzed by detection of fluorescence in a free R group of 5' terminal fluorescent probe[5-7]. However, the specificity of the target sequence is not that high and false-positivity remains a problem[8]. On the contrary, TB-IGRA is based on the principle that in TB patients, T lymphocytes would secrete interferon-γ under specific antigen stimulation, thus its sensitivity and specificity are higher than that of RT-PCR[9-11]. The main advantage of this method is that it can eliminate the interference caused by non-tuberculosis causes in patients and can be used for epidemiologic investigations. Our results also revealed that the combination of RT-PCR and TB-IGRA is superior to RT-PCR in the diagnosis of TB. So, by improving this combination, for example, optimizing the design of primers and PCR condition, we hope that this combination method can be implemented in clinical settings.

In conclusion, the RT-PCR detection of TB DNA combined with TB-IGRA is a good method for pulmonary TB diagnosis, and has practical significance in clinical pulmonary TB diagnosis, therefore its clinical application should be advocated and promoted.

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Sažetak

KLINIČKA PRIMJENA RT-PCR U OTKRIVANJU DNK TUBERKULOZE U KOMBINACIJI S TB-IGRA U DIJAGNOSTICI PLUĆNE TUBERKULOZE S NEGATIVNIM NALAZOM SPUTUMA

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Cilj istraživanja bio je ispitati otkrivanje DNK tuberkuloze u plućnom alveolarnom ispirku pomoću lančane reakcije polimeraze u stvarnom vremenu s fluorescentnim bojama (RT-PCR) u kombinaciji s kliničkom primjenom dijagnostike plućne tuberkuloze s negativnim nalazom sputuma pomoću testa otpuštanja TB interferona-γ (TB-IGRA). Od listopada 2014. do listopada 2015. godine nasumce su odabrane 632 osobe bolnički i izvanbolnički liječene u našoj bolnici, od kojih je 459 uključeno u ispitnu skupinu podvrgnutu otkrivanju pomoću RT-PCR u kombinaciji s testom TB-IGRA, a 173 u kontrolnu skupinu podvrgnutu elektroničkom otkrivanju u bronhoskopskom alveolarnom ispirku; rezultati su statistički obrađeni. Stopa pozitivnih nalaza bila je 96,51% u ispitnoj skupini, odnosno značajno viša od one u kontrolnoj skupini (66,47%), uz statistički značajnu razliku (χ^2=109,68; p=0,00). Stopa stvarno pozitivnih nalaza bila je 97,7% u ispitnoj skupini i 67,92% u kontrolnoj skupini, dakle značajno viša kod bolesnika ispitne skupine u usporedbi s kontrolnom skupinom, uz statistički značajnu razliku (χ^2=112,04; p=0,00). Osjetljivost i specifičnost, kao i Youdenov indeks bili su značajno viši u ispitnoj skupini u usporedbi s kontrolnom skupinom. U zaključku, otkrivanje TB DNK pomoću RT-PCR u kombinaciji s TB-IGRA vrlo je dobra metoda za dijagnosticiranje tuberkuloze, koja se može primjenjivati u kliničkoj dijagnostici plućne tuberkuloze.

Ključne riječi: *RT-PCR; TB-IGRA; Alveolarni ispirak; Dijagnostika*