Development and Evaluation of a Simple Latex Agglutination Test for the Detection of Pig Antibodies Against Human T-cell

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ABSTRACT

Based on human T-cell as a source of antigen, a simple and rapid latex agglutination test (LAT) was developed to detect pig antibodies against human T-cell instead of the time-consuming erythrocyte rosette inhibition test (ERIT). A total of 380 pig serum samples were subjected to the LAT and the sensitivity and the specificity were assessed in comparison with ERIT. The corresponding sensitivity and specificity were 92.05% and 98.61%.

INTRODUCTION

Immunosuppressants were used to prevent and cure the reject reaction in organ transplantation and in some hematology diseases (Scheinberg et al., 2009; ZHAN et al., 2010a; ZHAN (b) et al., 2010b). As an immunosuppressant, pig antibody (immunoglobulin) against human T-cell was widely used to improve long-term allograft survival by delaying the first episodes of acute rejection, and to treat acute rejection (Shidban et al., 2001; XIE, 2008; Wang et al., 2008). The standard serological assay to detect the titer of pig antibody against human T-cell is ERIT (Chinese Pharmacopoeia committee 2010a). The ERIT can directly detect the titer of the pig antibody against human T-cell, but it is labor-intensive, time-consuming, requires a continuous source of appropriate erythrocytes and occasionally results in errors. To avoid these limitations, a simple, rapid and cost low latex agglutination test was developed to detect the titers of antibodies against human T-cell from pig serum samples in the present study. LATs were widely be used in practice to detect antibodies (Guiping et al., 2010; Xiaojuan et al., 2005; Yang et al., 2010), bacterium identification (Narang et al., 2009; Sanz et al., 2010) and bacterium resistance (Qian et al., 2010). In the study, we have developed and valuated a simple, rapid, specific LAT for detection of antibodies against human T-cell.

MATERIALS AND METHODS

Cell and serum samples

Human T-cell suspension (5.0×106 T-cell per milliliter), anti-human-T-cell-positive

polyclonal sera (ERIT titer ≧1:1000), anti-human-T-cell-negative polyclonal sera (ERIT titer 0) and 380 serum samples were kindly provided by Hunan Lufeng Biotechnology Limited company. Anti-human-T-cell-positive polyclonal sera were prepared as described below. SPF pigs were injected with human T-cell weekly until its antibodies' titer was 1:1000 or more by ERIT. Anti-human-T-cell-negative polyclonal sera were collected from SPF pigs (ERIT titer 0), which had been injected with Hank's solution. Blood samples (n=380) were collected from healthy pigs according to the standard the document (Chinese Pharmacopoeia committee, 2005b) after injection (or not) with human T-cell suspension at the fatten stage. Blood samples were centrifuged at 3000 × g for 10 minutes for the separation of sera and stored at -20°C until used. Polystyrene beads (treated by trypsinase) were presented as a gift by Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University.

Erythrocyte rosette inhibition test

The ERIT was performed per the standard procedure (1), with minor modifications. Briefly, 100µl of anti-human-T-cell serum sample was added to an equal volume of human T-cell suspension, shaken and incubated at 37°Cwater for 30 minutes. One hundred microliters of 1% sheep erythrocyte suspension was added and mixed at room temperature for 15 minutes. After they were centrifuged at 500× g for 5 minutes, the mixed reagents were stored overnight at 4°C. The following morning, 100µl of TrypanBlue solution was added to the mixed reagents and shaken gently. The forming ratio of Rosette was calculated with a microscope. One hundred microliters of Hank's containing 20% fetal bovine serum instead of 100µl of anti-human-T-cell serum sample was prepared as a control according to the above process. The inhibition ratio of Rosette was calculated. The ERIT titer is the highest dilution of the serum samples in which the inhibition ratio is 25% or more.

Latex agglutination test

The optimized concentration of human T-cell suspension (antigen) to coat the latex beads and that of bovine serum albumin (BSA) in blocking the buffer were determined by the following processes. One hundred microliters of antigen was added to equal volume of 2% latex beads (polystyrene beads, 0.7-µm diameter; Medical Examination Institute of Shanghai, Shanghai, China). The solution was incubated at 37°C for 1h and then centrifuged at 5,000×g for 10 minutes. The supernatant was carefully aspirated out. The pellet was re-suspended in 1 ml of blocking buffer (10 mmol L-1 PBS, pH 7.4, containing 0.02% BSA) and centrifuged. The beads were washed more two times with the blocking buffer. After the final wash, the beads were re-suspended in 200 microliters of PBS. The washed

Table 1 *Optimal conditions for the LAT: determination for the concentrations of antigen and BSA by measuring spontaneous agglutination with PBS.*

BSA	Result of antigen dilution									
(%)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
1. 0	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-		-
0.09	-	-	+	-	-	-	-	-	-	-
0.06	-	+	+	-	-	-	-	-		-
0.03	+	+	+	+	-	-	-	-		-
0	++	+	+	+	-	-	-	-	-	-

Note: every crossing reagent mixture (BSA + antigen) reacts with PBS.
++, moderate agglutination; +, mild agglutination; -, no agglutination

antigen was serially diluted twofold with PBS (10mmol L-1, pH7.4) from 2-1 to 2-9 (Table 1). Each of the nine diluted antigens was mixed with each of the six blocking buffers, of which every BSA concentration was different (Table 1 and Table 2). Each mixed reagent was incubated overnight at 4°C. Every mixed reagent (15µl) was mixed with equal volume of PBS (Table 1) at room temperature to determine the optimal BSA concentration by measuring spontaneous agglutination. At the same time, every mixed reagent (15µl) was mixed with an equal volume of anti-human-T-cell-positive polyclonal serum (Table 2) to determine the optimal antigen concentration to coat latex bead by agglutination. The optimal conditions for coating latex beads were determined to be when strong agglutination with positive antiserum and no spontaneous agglutination with PBS are found in one mixed reagent in table 1 and table 2. The latex beads sensitized in the optimal condition were stored at 4°C until used.

The LAT was performed by adding 15 microliters of serum samples and an equal volume of sensitized latex beads to a clean glass slide. These reagents were gently mixed with sterile tips and left at room temperature for 3 minutes. In positive antisera, the agglutination was observed as formation of fine granular particles, settled at the edge of the reagent mixtures. If the suspension remained homogenous after 3 min, the test was declared negative. PBS and anti-human-T-cell-negative polyclonalsera were used as negative controls.

Comparison with ERIT

A positive serum sample (titer 1:1000) was serially diluted twofold with PBS (10mmol L-1, pH7.4) from 2-1 to 2-8 (Table 3). Each diluted serum sample was subjected to test by both of ERIT and LAT.

Sensitivity, specificity, reproducibility and stability of the LAT

A total of 380 pig serum samples were subjected to the LAT to determine its specificity, sensitivity and concordance in comparison with ERIT as described below (T. M. A. Senthilkumar et al., 2010) (Table 4).

Sensitivity = a / (a + c)×100%, where "a" is the number of serum samples positive by the LAT and ERIT, while "c" is the number of serum samples positive by ERIT, but negative by the LAT.

Specificity = d / (b + d)×100%, where "d" is the number of serum samples negative by LAT and ERIT, while "b" is the number of serum samples negative by ERIT, but positive by the LAT.

Concordance = a + d / (a + b + c + d)×100%

The reproducibility was assessed by latex beads sensitized with three batches of antigen preparations (same concentration) to evaluate a total of 100 serum samples. For

Table 2 *Optimal conditions for the LAT: determination for the concentrations of antigen and BSA by measuring agglutination with anti-human-T-cell-positive polyclonal sera.*

BSA	Result of antigen[a] dilution									
(%)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
1.0	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	+	+	-	-	-	
0.09	-	-	-	+	+	++	-	-	-	-
0.06	-	-	-	+	+	+++	+	-	-	-
0.03	-	-	+	++	+++	+++	++	++	-	-
0	-	-	-	+++	+++	++	++	++	++	-

Note: every crossing reagent mixture (BSA + antigen) reacts with anti-human-T-cell-positive polyclonal sera. +++, strong agglutination; ++, moderate agglutination; +, mild agglutination; -, no agglutination

Table 3 *The LAT in comparison with ERIT by positive serum sample (ERIT titer 1:1000)*

Dilute	1:1	1:2	1:4	1:8	1:6	1:32	1:64	1:128	1:256
Titer	1:1000	1:500	1:250	1:125	1:62.5	1:31.25	1:15.63	1:7.81	1:3.90
ERIT	pa	p	p	p	p	p	p	p	p
LAT	+++	+++	++	++	+	+	+	-	-

[a] positive: inhibition ratio was 25% or more by ERIT;
+++, strong agglutination; ++, moderate agglutination; +, mild agglutination; -, no agglutination

Table 4 *Correlation between the LAT and ERIT for the detection of 380 anti-human-T-cell serum samples.*

Result	No. of samples with ERIT result of		total
	positive	negative	
LAT positive	213 (a)	2 (b)	215
LAT negative	23 (c)	142 (d)	165
total	236	144	380

ERIT positive: inhibition ratio was 25% or more. LAT positive: strong agglutination (+++) and moderate agglutination (++).
Sensitivity of LAT: 90.25% (213 /236); Specificity of LAT: 98.61% (142 /144); Concordance: 93.42% ((213 + 142)/380).

the stability of the LAT, the sensitized latex beads stored at 4°C for 1, 2 and 3 month(s) were subjected to reactions with anti-human-T-cell-positive polyclonal serum.

RESULTS

Latex agglutination test

A simple and rapid latex agglutination test was developed using human T-cell as the antigen. The working dilutions of the blocked and sensitized latex beads reacting with either PBS or anti-human-T-cell-positive polyclonal serum are shown in Table 1 and Table 2. In detail, as can be seen from table 1, some sensitized latex beads displayed spontaneous agglutination with PBS while BSA concentration was 0%, 0.03%, 0.06% and 0.09%. In table 2, while the antigen was diluted to 1:16, 1:32 and 1:64, strong agglutination with positive antiserum was observed. So latex beads sensitized with antigen diluted at 1:32 (antigen concentration of 1.56×105 T-cell per milliliter) and no BSA in blocking buffer were suggested as the optimal condition for the assay in practice.

Comparison with ERIT

Each of these diluted positive serum samples (ERIT titer 1:1000) was subjected to testing by both ERIT and LAT. We observed that while the serum sample was diluted at 1:64 (ERIT titer 1:15), the diluted serum samples were detected positive. But they were detected negative at dilutions of 1:128 and 1:256 (ERIT titer were 1:7.8 and 1:3.9).

Sensitivity, specificity, reproducibility and stability of the LAT

Three hundred-eighty serum samples tested for antibodies by the LAT and ERIT. Among 380 serum samples, 236 samples were positive and 144 samples negative by ERIT. When 236 ERIT positive samples were tested by LAT, there were 213 samples positive and 23 samples negative. While 144 ERIT negative samples were tested by LAT, there were 142 samples positive and 2 samples negative. In comparison with ERIT, the sensitivity of the LAT is 90.25% and its specificity is 98.61%. The concordance between the two tests was 93.42% (Table 4).

There were 96, 95, and 95 samples tested positive by the three batches, indicating a high level of reproducibility. For the stability of the LAT, similar agglutination sensi-

tivities were achieved with sensitized latex beads stored at 4°C for 1, 2 and 3 months.

DISCUSSION

The purpose of this study is to develop a simple, rapid, cost low and on-site method to detect anti-human-T-cell antibodies as an alternative to ERIT. The LAT requires human T-cell as a source of antigen. In the present study, when sensitized by highly concentrated antigen, the latex beads can react with both PBS and positive antibodies. Normally, spontaneous agglutination with PBS and strong reactivity with anti-human-T-cell -positive serum can be observed when antigens of high concentration are used. But in this study, a weak, spontaneous agglutination was visible with PBS when the antigen was diluted from 1:2 to 1:16. At the same time, no agglutination was visible with anti-human-T-cell-positive serum when the antigen was diluted from 1:2 to 1:4. Further investigation is needed to determine the reason for this.

The LAT was also found to be sensitive until the anti-human-T-cell-positive serum was diluted to 1:15 (titered by ERIT). This LAT is simple, rapid and easy to operate on site, with no special equipment or skilled personnel required. Additionally, results can be obtained within three minutes. Although the sensitivity of the LAT is lower than that of ERIT, the LAT avoids costly equipment and requires minimal laboratory facilities. Thus, the LAT has practical applications. For example, the titers of anti-human-T-cell antibodies used in preventing and curing the rejection reaction in organ transplantation are required 1:1000 or more by ERIT (Chinese Pharmacopoeia committee, 2005). When detecting their titers, the antisera are generally diluted to 1:1000 and then tested by ERIT. If their inhibition ratio by ERIT is 25% or more, these antisera are up to standard and can be used in practice. But this ERIT test requires 24 hours or more to detect the titers. Now, we can use the LAT to determine whether these antisera are up to standard (ERIT titer $\geq$1:1000) as below. For example, antisera are diluted (1:64) according to Table 3 and then tested by LAT. If positive by LAT, their titer by ERIT must be 1:1000 or more and they must be up to standard. If negative by LAT, those antisera need further testing by ERIT to determine whether they are up to standard. In fact, the titers of most prepared antisera are 1:1000 or more, so the LAT can be used to screen most antisera and only a few need further testing by ERIT. This method would save a considerable amount of time and labor. In the present study, the sensitivity and specificity of LAT were observed as 90.25% and 98.61%, respectively, as compared with ERIT. Concordance (93.42%) showed perfect agreement between the two tests.

Overall, the results of our study show the generation of a simple, cost-effective, sensitive and specific latex agglutination test. It has great potential in the monitoring of antibodies against human T-cell. This method may also be employed in practice.

CONCLUSION

A simple, rapid, practical and cost-effective alternative to ERIT was developed to detect the antisera against human T-cell.

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