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Detection of *Theileria sergenti* in Bovine Lymphocyte by Polymerase Chain Reaction

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PCR 기법을 이용한 소 Lymphocyte 내 *Theileria sergenti*의 檢出

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요 약 : 소 림프구내의 *Theileria sergenti* 감염을 확인하기 위하여 *T. sergenti* 감염혈액에서 림프구를 분리한 후 중합효소연쇄반응을 실시하였다. 또한, 분리한 림프구내의 *T. sergenti* 감염을 증명하기 위하여 IFA test와 acridine orange stain을 실시하였다. 그 결과 다음과 같은 성적을 얻을 수 있었다. *T. sergenti* 감염혈액의 전혈과 림프구를 각각 생리식염수로 2배율 연속희석하여 중합효소연쇄반응을 실시한 결과, 림프구내에서는 1,024배 희석배율까지 *T. sergenti*의 genomic DNA가 증폭되었으며, 전혈내에서는 256배 희석배율까지 증폭되었다. 그리고 중합효소연쇄반응으로 *T. sergenti* 감염이 확인된 림프구를 이용하여 IFA test와 acridine orange 염색을 실시한 결과, 림프구내에 *T. sergenti*가 존재하는 것을 증명할 수 있었다. 한편, 중합효소연쇄반응을 이용한 림프구내의 *T. sergenti* 감염의 진단 유용성을 확인하기 위하여 전북지역에서 사육중인 소 16두를 대상으로 이들의 혈액으로 PCR 증폭을 실시하였다. 그 결과 전혈에서 genomic DNA를 취한 경우에는 3두(18.8%)만이, 그리고 림프구에서 genomic DNA를 취한 경우에는 11두(68.8%)의 소에서 *T. sergenti* DNA의 증폭을 관찰할 수 있었다.

Key word **:** *Theileria sergenti*, lymphocytes, PCR

Introduction

T. sergenti, a protozoan parasite transmitted by the vector tick, *Heamaphysalis longicornis*, causing anemia, pyrexia, retarded growth and reduced milk production in cattle of Korea[1,2]. When the parasitemia of *T. sergenti* is lower or latent, *T. sergenti* was difficult to distinguish from other parasite such as anaplasma and babesia etc[3]. If those of latent *T. sergenti* infected cattle were inflicted to stress or other-infection, they become severe anemia, icterus and pyrexia or die[4,5].

Generally, the detection of *T. sergenti* were focused on merozoite which intra-erythrocytic stage by Giemsa' stain[1,5,6]. But the detection was difficult when in lower parasitemia or latent infection[3].

Presently, serodiagnostic methods by fluorescent-antibody[7-10], southern hybridization by isotope[11,12] and PCR amplification[13-19] are used for the diagnosis of theileriosis. Those method reported frequently for the diagnosis of *T. sergenti* merozoite which in bovine intra-erythrocytic stage, but not in bovine lymphoid tissue or in tick stage which schizont or gametogony etc[20].

Therefore, we carried out PCR amplification with lymphocytes which separated from *T. sergenti* infected whole blood to identify the detection of *T. sergenti* infection in lymphocytes. As the results, *T. sergenti* in lymphocytes was identified by PCR amplification as well as by IFA test and acridin orange stain. So, we would like to report about the detection *T. sergenti* infection in lymphocytes by PCR.

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Materials and Methods

Separation of lymphocyte

Lymphocytes were separated by the procedure of Barta[1]. In brief, *T. sergenti* infected whole blood were mixed with RPMI 1640(SIGMA) at room temperature. This solution were carefully delivered onto the separating medium(Ficoll-hypaque, d=1.083), and centrifuged for 40 min at 600 g. After centrifugation, the lymphocytes in the interface between plasma and cell-separating medium were harvested and used in the present study.

Acridine orange stain and IFA test

To detect the *T. sergenti* in lymphocyte and whole blood, acridine orange stain and IFA test were carried out. IFA test was undertaken by the procedure of Nam *et al*[7]. In brief, lymphocytes were fixed in cold acetone and reacted with FITC conjugated anti-*T. sergenti* anti IgG. Also, acridine orange stain was undertaken by the modified procedure of Gainer[3].

Polymerase Chain Reaction

Isolation of template DNA for PCR: Template DNA for PCR were isolated from whole blood and lymphocytes by the procedures of kawasaki *et al*[5] and Matsuba *et al*[6].

Optimization of PCR condition: To determine the optimal concentration of *Taq* polymerase enzyme and PCR cycle, PCR amplification was carried out as shown Table 1.

Table 1. Optimization of *Taq* polymerase enzyme concentration and cycle number in PCR mixture

Reagent	1	2*	3	4
Upper primer (10 pmol/μl)	1.2	1.2	1.2	1.2
Lower primer(10 pmol/μl)	1.2	1.2	1.2	1.2
dNTP(2.5 mM)	1.2	1.2	1.2	1.2
10× PCR buffer	3	3	3	3
Taq polymerase(5 U/μl)	0.5	0.75	0.5	0.75
H.O	19.9	19.65	19.9	19.65
Template	3	3	3	3
Total Volume	30	30	30	30
Cycle Number	35	35	40	40

*Optimized PCR condition

PCR amplification: PCR amplification was carried out by modified procedure of Chae *et al*[2]. The KTS1R primers used this experiment had been manufactured according to the sequence of *T. sergenti* merozoite genomic DNA that separated from infected erythrocytes: KTS1R upper primer(5'-CCTCTTGAAGT CATCCATGT-3', nucleotide position 48 and the KTS 1R lower primer(5'-CA CTGAGCTGGAAAGAGC-TA-3', nucleotide position 156). The final reaction volume was adjusted to 30 μl including 1.2 μl of each primer(10 pmol/μl), 1.2 μl of 2.5 mM dNTP, 3 μl of 10X PCR buffer, 0.75 IU of *Taq* polymerase enzyme and 3 μl of template DNA, and run in a DNA thermal cycler(BARNSTEAD/Thermolyne Co, USA) through 35 cycle: 96℃ for 30 sec(denaturation), 60℃ for 1 min(annealing), 72℃ for 1 min(polymerization).

Analysis of DNA product: The produced DNA(5 or 10 μl) were fractionated by 1.5% agarose or 10% polyacrylamide gel-electrophoresis. The DNA was visualized by ethidium bromide staining and UV transilluminator. 10 or 100 bp DNA ladder(Gibco BRL, U.S.A.) were used for the size marker. *T. sergenti* probe were used for the positive control, and uninfected bovine kidney cell genomic DNA were used for negative control of PCR amplification.

Result

Polymerase Chain Reaction

Optimization enzyme concentration and PCR cycle: As shown Table 1, the optimal PCR condition were 35 cycle and 0.75 IU of *Taq* polymerase enzyme in 30 μl of final volume(Fig 1).

Determine the detection sensitivity by PCR amplification: Lymphocytes and whole blood infected with *T. sergenti* were 2 fold serially diluted by 0.9% NaCl, and those were PCR amplified. *T. sergenti* were detected higher in dilution of lymphocytes (1024 fold) than in those of whole blood(256 fold) (Fig 2).

For comparision of the detection rate between whole blood and lymphocytes, we carried out PCR amplification to 16 cases of Korean cattle. Of those, 11 cases were positive in lymphocyte and 3 cases were positive in whole blood by PCR amplification.

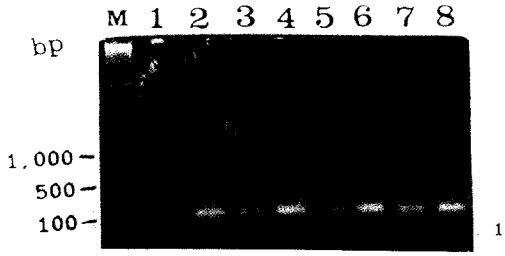

Fig 1. Optimization of PCR cycle and tag polymerase enzyme concentration in PCR condition. M; Marker(1 kb ladder), Lane 1, 3, 5, 7 are prepared from bovine blood. Lane 2, 4, 6, 8 are prepared from separeted lymphocytes.

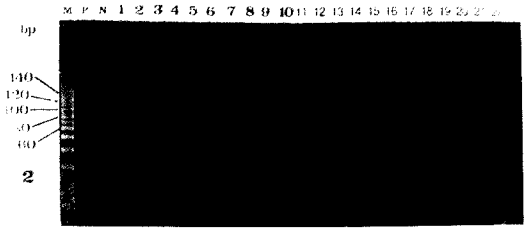

Fig 2. Determination of the sensitivity of the *T. sergenti* in bovine peripheral blood and separated lymphocyte by PCR. M; Marker(10 bp ladder), P; Positive control with KTS1 DNA, N; Negative control with noninfected bovine kidney cell DNA. Lane 1~10 are prepared from 2 folds serial dilution of bovine blood. Lane 11~22 are from 2 folds serial dilution of separated lymphocytes

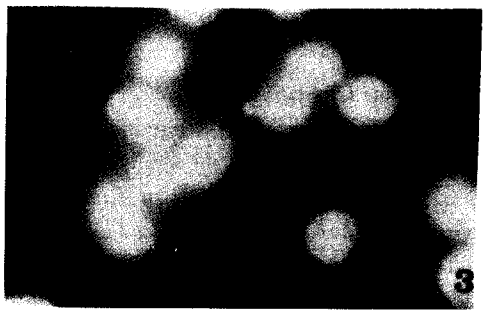

Fig 3. *T. sergenti* in lymphocytes from bovine peripheral blood (acridine orange stain × 1,000).

Acridine orange stain

T. sergenti in lymphocytes and whole blood were identified by acridine orange stain(Fig. 3, 4).

IFA test

T. sergenti in lymphocytes showed a specific reaction against FITC conjugated anti-*T. sergenti* anti IgG(Fig. 5).

Fig 4. *T. sergenti* in erythrocytes from bovine peripheral blood (acridine orange stain × 1,000).

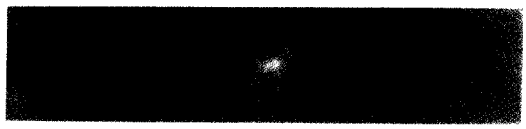

Fig 5. *T. sergenti* in separated lymphocytes reacted with positive serum of the infected bovine (IFA test × 1,000).

Discussion

Generally, Giemsa's[3,10] or Diff-quick stain were used for diagnosis of theileriosis. And serodiagnostic method[4,11,15,16] including IFA test and ELISA were also used. Recently, Bishop *et al*[9] and Tanaka *et al*[10] reported the southern hybridization and PCR are sensitive method for the diagnosis of theileriosis. But those method focused upon the diagnosis of *T. sergenti* merozoite which in bovine intra-erythrocytic stage[2,9,10,15], not in bovine lymphoid tissue or in tick stage which schizont or gametogony etc.

Although Kawazu *et al*[20] reported the diagnosis of *T. sergenti* in lymphoid tissue by using IFA, the diagnosis of *T. sergenti* in peripheral blood lymphocytes by PCR is not reported yet.

Therfore, to determine the sensitivity of detection *T. sergenti* in lymphocytes by PCR amplification compared with usual methods, lymphocytes and whole blood were two-fold serially diluted in 0.9% NaCl. *T. sergenti* were detected higher in dilution of lymphocytes(1024 fold) than in those of whole blood (256 fold). Therefore, we could find out that the detection of *T. sergenti* in lymphocyte is more sen-

sitive than whole blood.

Also, IFA test, acridine orange and Diff-quick stain were carried out for the credibility of *T. sergenti* infection of lymphocyte by microscopic observation. *T. sergenti* in lymphocyte were detected by IFA test and acridine orange stain, but not detected by Diff-quick stain.

From these results, the examination of *T. sergenti* in lymphocyte by PCR is sensitive and accurate method to diagnosis of bovine theileriosis.

Conclusion

We carried out PCR amplification with lymphocytes separated from whole blood of *T. sergenti* infected cattle to identify the detection of *T. sergenti* in lymphocytes. *T. sergenti* in lymphocytes was identified by PCR amplification as well as by IFA test and acridine orage stain. And the detection of *T. sergenti* in lymphocytes is more sensitive than whole blood by PCR.

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