大韓바이러스學會誌 : 第 26 卷 第 2 號 1996
J. Korean Soc. Virology, Vol. 26, No. 2, 1996

Use of Nested Polymerase Chain Reaction for Identification of *Rickettsia tsutsugamushi* Serotype Cultured in Human Embryonic Lung Cells

Chang-Nam An, Gyu-Jin Woo[1], Tae-Yeon Kim[1], Kwang-Soon Shin[2], Chul-Joong Kim[2] and Luck-Ju Baek[3]

Central Research Institute, Korea Green Cross Corporation, Seoul, Korea
[1]Mogam Biotechnology Research Institute, Seoul, Korea
[2]Department of Veterinary Preventive Medicine, College of Veterinary Medicine, Chungnam National University, Taejon, Korea
[3]Department of Microbiology, College of Medicine, Korea University, Seoul, Korea

=국문초록=

Nested PCR을 이용한 사람 유래 태아 폐세포에서 배양된 *Rickettsia tsutsugamushi*의 혈청형 동정

녹십자 종합연구소, [1]목암 생명공학연구소, [2]충남대학교 수의과대학 전염병학교실, [3]고려대학교 의과대학 미생물학교실

안창남 · 우규진[1] · 김태연[1] · 신광순[2] · 김철중[2] · 백락주[3]

*Rickettsia tsutsugamushi*의 원형균주인 Karp주와 Gilliam주를 초대 배양된 사람 정상 2배체 폐세포 (LuMA cell)를 이용하여 증식과 세포병변들의 속도를 비교할 수 있었고, 배양된 균주는 네스티드 프라이머를 사용하여 혈청형을 동정할 수 있었다. *R. tsutsugamushi*의 세포벽 외막에 존재하며 혈청형을 결정하는 주요항원은 54-56Kd 단백인 것으로 밝혀지고 있는데, 이 단백 유전자의 DNA 염기서열을 분석하여 Karp주와 Gilliam주의 공통서열로 첫번째 프라이머쌍을 만들었고 첫번째 프라이머쌍의 안쪽에 위치한 혈청형 사이에 차이가 있는 서열로 두번째 프라이머쌍을 만들었다. 네스티드 뉴클레오티드 프라이머는 중합효소 연쇄반응의 특이성을 증가시킬 수 있는데 이 실험 결과로 이 PCR방법은 scrub typhus의 진단과 혈청형의 동정에 적용될 수 있을 것으로 보여진다.

Key Words: Nested PCR, *Rickettsia tsutsugamushi.*

INTRODUCTION

Rickettsia tsutsugamushi is a gram-negative bacterium and obligately intracellular parasite [7, 32] which causes the human disease scrub typhus [4, 5]. The antigenic heterogeneity [11] of strains of scrub typhus rickettsiae isolated from infected human [8], animal [21], and chigger sources [9, 21] has been well documented by means of the complement fixation test [2], plaque reduction assay [1, 25], and immunofluorescence tests [3, 20, 28]. The Karp and Gilliam strains of *R. tsutsugamushi* grow well in embryonated eggs [22] or cell culture [17, 19, 23]. Procedures of tissue culture for rickettsiae are similar to those used in virology, except that the use of antibiotics must be carefully avoided and, in some experiments, the monolayers must be maintained for long periods of time. Maximum intracellular growth of the rickettsiae occurred only in the presence of com-

plex media capable of supporting proliferation of host cells. Plaque formation by *R. tsutsugamushi* has been reported in chicken embryo (CE) cells, Vero cells, and irradiated L-929 cells [31]. Recently, we have established an excellent cell line, human embryonic lung cells [6] (LuMA cell), for *R. tsutsugamushi* production.

Humans infected with scrub typhus rickettsiae produce serum antibodies to at least eight rickettsial specific protein, including the 110, 58, 56 and 47Kd polypeptide. Among these scrub typhus antigens [16], the 56Kd which is major outer membrane protein and 58Kd protein [29] are major proteins in this rickettsia and are the antigens recognized by infected animals and human. Two antigens about 110Kd and 56Kd protein [24, 26, 27] have strain-variable molecular weights, contain strain-specific epitopes, and are possibly involved in the strain-specific [12] protective immune response.

Recently, application of polymerase chain reaction (PCR) [13] to the diagnosis of infectious diseases has been reported. This method is very effective in case of difficulty of immunological technique and isolation of the causative agent. Here, we describe the propagation of *R. tsutsugamushi* in human embryonic lung cells and the use of nested PCR for identification of rickettsial serotypes. The primers used for PCR were designed on the basis of the DNA sequence of the gene encoding the 56Kd antigen, and serotype-specific primers were used in the second PCR amplification.

MATERIALS AND METHODS

Rickettsial seeds and cell culture

The rickettsiae employed were *R. tsutsugamushi* Karp strain, with a history of 56 passages in embryonated eggs (56EP) and Gilliam strain, with a history of 169 passages in embryonated eggs (169EP). All rickettsiae were cultured in LuMA cells. LuMA cells were obtained from Mogam Biotechnology Research Institute (MBRI). These cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS). After inoculation of *R. tsutsugamushi*, maintenance medium containing 2% FBS was used. LuMA cells were propagated as monolayer cultures at 37℃ in a humidified atmosphere of 5% CO_2 in air. Rickettsial suspensions was added to each monolayer after the growth medium had been removed. The flasks were held at room temperature for 1 hour, with frequent rocking to dis-

Table 1. The sequences of oligonucleotide primers used for lst PCR

Oligonucleotide primer	Sequence	Region of nucleotide sequence
RT1	5′ CTTACACCACCTCAGCCTACT3′	Karp 396-416 Gilliam 399-419
RT2	5′ GTCACTTAATACTTTGACAGG3′	Karp 777-797 Gilliam 753-773

Table 2. The sequences of the oligonucleotide primers used for nested PCR

Oligonucleotide primer	Sequence	Region of nucleotide sequence
OLIGO KT1	5′ ATTCCTAACCAGACCTCAGCA3′	459-479
OLIGO KT2	5′ AACCATAGGCCCATTAGGATC3′	588-608
OLIGO RTI	5′ CTTACACCACCTCAGCCTACT3′	399-419
OLIGO GT2	5′ ATTTTGCCGAGGTCTAGGCTG3′	648-668

```
Karp     -555                                                                                                         AAG CTT   -550
Gilliam  -555                                                                                                         --- ---   -550

Karp     -549 GTT  CAT TTT TTA TGT GGG CTA ATT TTA GAT AAT GCA ATG TTA GTA TAA TT   -500
Gilliam  -549 ---  --- --- --- -A- --- --- --- --- --- --- A-- --- --- --- --- --   -500

Karp     -499 A TGT GGT TAA TTA ATG TAT CTT GAT TTA AGA TTT TAT ATA AAT ATA ATA A   -450
                                        -35                         -10
Gilliam  -499 - --- --- --- --- --- --- --- --- --- --- --- --- --- --A TAT -AT -   -450

Karp     -449 GA  TTT ATG TAG GGC TTA ATT ATT AGC TTA AAA AAC TGT TGC TAT TTT AGC   -400
Gilliam  -449 AG  -T- --G CTT -AT -AT TAG C-- GAA AA- CTG TTG CTA -TT --G C-A -AA   -400

Karp     -399 TAA AAA TAA AAG TTT GGG CAA GAA AAA TTA TTA ATA ATT GAA GGT AGT TG   -350
Gilliam  -399 AT- --C -TT T-- GCA A-A A-- ATT -TT AAT AAT TG- -GG T-G TTG TTG C-   -350

Karp     -349 T TGC GTA AAA AGC TGT GTT ATG CTA TCT AAG GTT AAA TGT AGC AAG ATG C   -300
Gilliam  -349 - AAA AAG TGG T-T -A- -C- --C TA- GG- T-A C-G T-- CAA T-T GCT AAT A   -300

Karp     -299 TA ATA GAT AAT TAA TGT ATT TTC GAA CGT GTC TTT AAG CTA TAT ATA AGA   -250
Gilliam  -299 G- TA- TTA -TG --T -T- CGA ACG TGT -T- TAA GCA T-T A-- --A GAG CAG   -250

Karp     -249 GCA GTA TTC TAT TGA ATA TTG TTT CTA AGT ATA TAA AAA ATA AAA ATA CA   -200
Gilliam  -249 TGT TCT A-T G-A --T TGT C-T -AA G-T -TA TAT A-- --- TA- --- TA- AT   -200

Karp     -199 T TTT ACA ATT GAT AAA ACG CTT TGA GCA CAT TTT TAA CAC AGT GTT TTA T   -150
Gilliam  -199 - --A CA- TGG ATA --- CGC T-- GA- -CG ATA --- -T- ACA CAG TG- --T A   -150

Karp     -149 AG ATT GTT TAA ATT ATT TTA CAA GTA CTA TTA AAT ATT AGT ATA CTA AAT   -100
Gilliam  -149 TA GA- TG- -T- -A- TA- --T AC- AGT ACT A-T --A TA- TAG TAT ACT --G   -100
                                    -35                                -10
Karp      -99 AAT AGT TTT TTG ATA TAA AAC TAA AGT TAG TGT GGC TAA ATA ATT AGT TT    -50
Gilliam   -99 T-A TAG --- --T GAT AT- --A GT- --- --- --- --- --- --- --- --- --    -50

Karp      -49 A GAA TGG TTA CCA CTA AAA AAT AAA TTT AAT TCT TTT AAG GAG ATT AGA     -1
                                                                 RBS
Gilliam   -49 - --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---     -1
                                                                 RBS

Karp
sta56→      0 ATG AAA AAA ATT ATG TTA ATT GCT AGT GCA ATG TCT GCG TTG TCG TTG CC    49
Gilliam
tsg56→      0 --- --- --- --- --- --- --- --- --- --- --- --- --A --- --A --- --    49

Karp       50 A TTT TCA GCT AGT GCA ATA GAA TTG GGG GAA GAA GGA TTA GAG TGT GGT C    99
Gilliam    50 G --- --- --- --- --- --- --- --- --T --G --- GGA --- --- --- --- -   102

Karp      100 CT TAT GCT AAA GTT GGA GTT GTT GGA GGA ATG ATT ACT GGC GTA GAA TCT   149
Gilliam   103 -- --C -G- --- --- --- A-C --- --- --- --- --- --- --T -C- --- ---   152

Karp      150 GCT CGC TTG GAT CCA GCT GAT GCT GAA GGC AAA AAA CAC TTG TCA TTA AC   199
Gilliam   153 A-- --- --- --- T-- A-- --- T-- --G --A --- --- --T --- --- --- --   202

Karp      200 A AAT GGG CTG CCA TTT GGT GGA ACG TTG GCT GCA GGT ATG ACA ATC GCT C   249
Gilliam   203 - -C- --A --- --- --- --- --T --A --A --A --- --G --- --- --T --A -   252

Karp      250 CA GGA TTT AGA GCA GAG ATA GGT GTT ATG TAC CTT ACA AAT ATA ACT GCT   299
Gilliam   253 -- --- --- --- --- --- C-- --- --- --- --- --- -G- --- --- -GC ---   302

Karp      300 CAG GTT GAA GAA GGT AAA GTT AAG GCA GAT TCT GTA GGT GAG ACA AAG GC   349
Gilliam   303 G-- --- --- G-A --- --- -GC --- -T- --- --- AA- --- --- -T- --- --   352
```

Fig. 1.

```
Karp     350 A GAT TCT GTA GGT GGG AAA GAT GCT CCT ATA CGT AAG CGG TTT AAA CTT A       399
Gilliam  353 - --- --- -G- --- --- -C- --- A-- --- --- --- --- --- --- --- --- -       402

Karp     400 CA CCT CCT CAG CCT ACT ATA ATG CCT ATA AGT ATA GCT GTA CGT GAC TTT         449
Gilliam  403 -- --A --- --- --- --- --- --- --- --- --- --- --- -AT --- --T G-G        452

Karp     450 GGG ATT GAT ATT CCT AAC CAG ACC TCA GCA GCA AGC ACA AGC CGC AGC CT       499
Gilliam  453 --- G-- --- -C- GA- -TT -TT G-T CA- --T             G-T GCT G-G CAA -C   496

Karp     500 C AGG CTT AAT GAT GAG CAA CGT GCT GCA GCT AGG ATC GCT TGG TTA AAG A      549
Gilliam  497 A CA- --- -C- -T- --- --G --G --- --- -A- --- --T --- --- --G --- -      546

Karp     550 AT TGT GCT GGT ATT GAC TAT AGG GTA AAA AAC CCT AAT GAT CCT AAT GGG       599
Gilliam  547 -- -A- --- --- --- --- --- -T- --C CC- G-T --- C-G A--     CC- AAT       593

Karp     600 CCT ATG GTT ATA AAT CCG ATA TTG TTA AAT ATT CCA CAG GGT AAC CCT AA       649
Gilliam  594 G-- -GA --- --- --- --T G-- --- --- --- --- A-T --A --G CCA --- --       643

Karp     650 T CCT GTT GGA AAT CCA CCG CAG CGA GCA AAT CCG CCT GCA GGT TTT GCG A      699
Gilliam  644 - GTA CAG CCT -GA --T -G- --A                         AA- C-- -AC -     675

Karp     700 TA CAT AAC CAT GAG CAA TGG AGG CAT TTG GTA GTT GGG CTT GCT GCA TTA       749
Gilliam  676 -- -T- G-- --- -GT --G --- --A --- --- --- --- --T G-- A-- --- --G       725

Karp     750 TCA AAT GCT AAT AAA CCT AGC GCT TCT CCT GTC AAA GTA TTA AGT GAT AA       799
Gilliam  726 --- C-- --- --- --- --- --- -T- A-- --- --- --- --- --- --- --C --       775

Karp     800 A ATT ACT CAG ATA TAT AGT GAT ATA AAG CAT TTG GCT GAT ATA GCT GGT A      849
Gilliam  776 - --- --- A-- --- --- --- --- --- --- -CA --T --- --- --- --- --- -      825

Karp     850 TT GAT GTT CCT GAT ACT AGT TTG CCT AAT AGT GCA TCT GTC GAA CAG ATA       899
Gilliam  826 -- --- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- ---       875

Karp     900 CAG AAT AAA ATG CAA GAA TTA AAC GAT CTA TTG GAA GAG CTC AGA GAA TC       949
Gilliam  876 --- -G- --- --- --- --- --- --- --- G-- --- --- --C --- --- --T --       925

Karp     950 T TTT GAT GGG TAT CTT GGT GGT AAT GCT TTT GCT AAT CAG ATA CAG TTG A      999
Gilliam  926 - --- --- --- --- A-G ---     --- --- --- --- --- --- --- --- --- -      972

Karp     1000 AT TTT GTC ATG CCG CAG CAA GCA CAG CAG CAG     GGG CAA GGG CAG CAA CAG  1049
Gilliam  973 -- --- --- --- --- --- --- --- --- --- --- CAG --- --- --- --- --- ---  1025

Karp     1050 CAA GCT CAA GCT ACA GCG CAA GAA GCA GTA GCA GCA GCA GCT GTT AGG CT      1099
Gilliam  1026 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --      1075

Karp     1100 T TTA AAT GGC AAT GAT CAG ATT GCG CAG TTA TAT AAA GAT CTT GTT AAA T     1149
Gilliam  1076 - --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -     1125

Karp     1150 TG CAG CGT CAT GCA GGA ATT AAG AAA GCG ATG GAA AAA TTA GCT GCC CAA      1199
Gilliam  1126 -- --- --- --- --- --- G-- --- --- --C --- --- --- --- --- --- ---      1175

Karp     1200 CAA GAA GAA GAT GCA AAG AAT CAA GGT GAA GGT GAC TGC AAG CAG CAA CA      1249
Gilliam  1176 --- --- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --      1225

Karp     1300 AT CTG AGT ATG ATT GTC GGC CAA GTT AAA CTC TAT GCT GAC GTA ATG ATA      1349
Gilliam  1276 -- --- --- --- --- -T- --- --- --- --- --- --- --- --- T-- T-T -C-      1325
```

Fig. 1.

```
Karp      1350 ACT GAA TCA GTC TCA ATA TAT GCT GGT GTT GGT GCA GGG TTA GCT TAT AC  1399
Gilliam   1326 --- --- --- T-- --- --- --- --- --- --- --- --- --- --- --- C-- --  1375

Karp      1400 T TCT GGA AAA ATA GAT AAT AAG GAT ATT AAA GGG CAT ACA GGC ATG GTT G  1449
Gilliam   1376 - -A- --- --- --- --- G-- --- --- --- --- --- --- --- --- --- --- -  1425

Karp      1450 CA TCA GGA GCA CTT GGT GTA GCA ATT AAT GCT GCT GAA GGT GTG TAT GTG  1499
Gilliam   1426 -- --- --- --- --- --- --- --- --- --- --- --- --G --- --A --- ---  1475

Karp      1500 GAC ATA GAA GGT AGT TAT ATG TAC TCA TTC AGT AAA ATA GAA GAG AAG TA  1599
Gilliam   1476 --- T-- --- --- --- --- --- C-- --- --C --- --- --- --- --- --- --  1575

Karp      1600 TT TTT CTT TAA AAT TAT AAA AAA AGC AGC TAA AAG TTC TTT ACA GGG TTT  1649
Gilliam   1576 -- --C T-- A-- -T- ATA --- --- -AG CAG CT- --A G-T C-- TAC A-- G--  1625

Karp      1650 TTA GCT GCT TTT TCA GAG TTT TTT TAT AAT AAT AAA AAT AAC TTT ATT CT  1699
Gilliam   1626 --T AGC TGC --- -TC AGA G-- --- -TA T-A T-A T-A --A TTA AC- TTA T-  1675

Karp      1700 T TGC TAT TTA ATT AGC TTG AAG CTT                                  1724
Gilliam   1676 C -TT GC- A-T TAA TTA GCT T-A AGC TT                               1702
```

Fig. 1. The nucleotide sequences of the *R. tsutsugamushi* Karp *sta56* gene and Gilliam *tsg56* gene including their flanking reions. The first base of the presumed *sta56* gene and tsg56 gene initiation codons are numbered 0. The presumed initiation codon for the *sta56* protein and *tsg56* protein are denoted by a rightward arrow (5' to 3'). Sequence resembling the consensus sequences for ribosomal binding sites (RBS) and promoter -10 and -35 regions are underlined. (From Stover, C.K. et al., (1990), *Infect Immun.* 58, 2076-2084 and Ohashi N. et al., [1990], *Gene.* 91, 119-122).

tribute the inocula. The infected monolayers were washed twice with Earle's balanced salt solution, growth medium was added and the flasks were incubated at 34℃. The infected cells were harvested after 9-11 days.

Plaque formation

Rickettsial stocks were assayed using this method and the results were expressed as the titer in plaque forming units (PFU)/ml. The plaque assay procedure used in this study was the same as that described in more detail in an earlier report [34]. The infected monolayers were then covered with an overlay of maintenance medium containing agarose at a final concentration of 0.5%. All of the infected monolayers were incubated at 34℃. A Second overlay was placed over the initial overlay after 7 days, and incubating at 34℃ was continued for an additional 10 days. Plaques were stained by an overlay containing 1% neutral red.

Preparation of template DNAs

Monolayers of infected cells in 150cm^2 plastic tissue culture flasks (Costar, Cambridge, MA) were homogenized with a Dounce homogenizer in 5 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA (TE buffer), and the DNA was extracted from homogenate supernatants obtained after centrifugation at 300g for 10 minutes. To extract DNA preparation, rickettsial suspensions were mixed with 1/10 volume of 10% sodium dodecyl sulfate (SDS) (final concentration of SDS, 1%) and incubated at 4℃ for 16 hours. After the addition of 1/10 volume of 10-fold-concentrated TE buffer, the mixture was further incubated with 3x crystallized chicken egg white lysozyme (Sigma Chemical Co., St. Louis, MO.) at a final concentration of 2 mg/ml for 30 minutes in an ice bath and then with proteinase K at a final concentration of 0.2 mg/ml for 1 hour at 55℃. The DNA in this lysate was purified by three extractions with an equal volume of a phenol-chloroform (1:1) mixture, after the addition of 1/10 volume of 3 M sodium acetate and followed by precipitation with 2.5 volumes of ab-

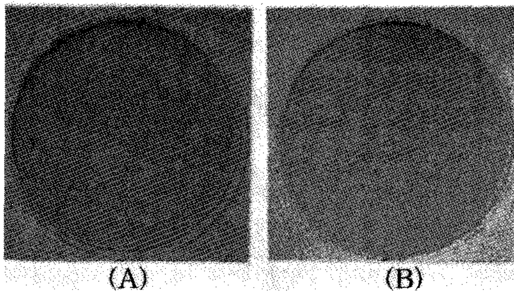

Fig. 2. *Rickettsia tsutsugamushi* plaques in LuMA cell culture. (A) Karp strain; (B) Gilliam strain.

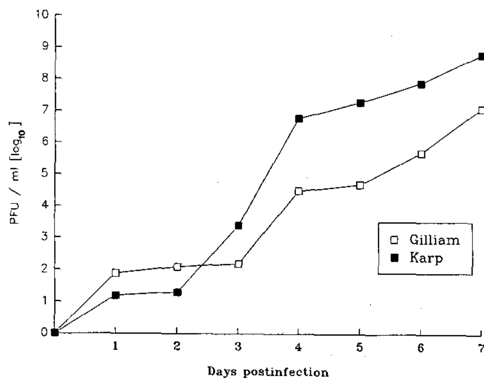

Fig. 3. Multiplication curves in LuMA cell culture infected with *R. tsutsugamushi* propagated in embryonated egg yolk sac after different incubation periods.

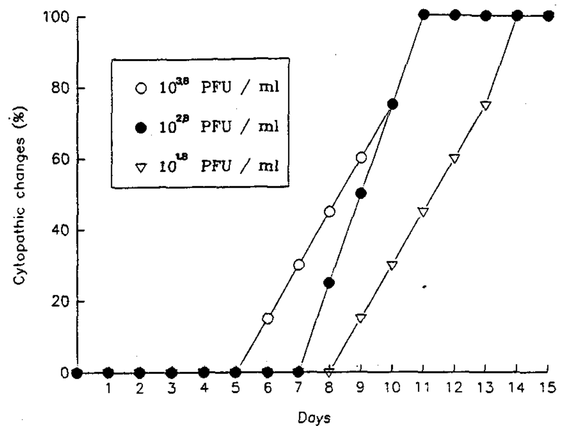

Fig. 4. Cytopathic changes in LuMA cell culture infcted with varying quantities of Karp strain of *R. tsutsugamushi* propagated in embryonated egg yolk sac.

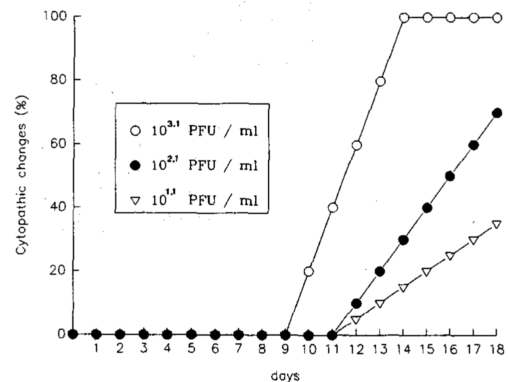

Fig. 5. Cytopatich changes in LuMA cell culture infected with varying quantities of Gilliam strain of *R. tsutsugamushi* propagated in embryonated egg yolk sac.

solute ethanol and resuspension in 50μl of TE buffer.

Oligonucleotide primers for PCR

Oligonucleotide primers for PCR were provided by Korea biotech. Inc. (Table 1). The primers used for 1st PCR were designed from the DNA sequence of the gene encoding the 56Kd antigen [30] of the Karp and Gilliam strains (Fig. 1).

Oligonucleotide primers for nested PCR

Oligonucleotide primers for nested PCR were obtained from Korea biotech. Inc. (Table 2).

PCR

The reaction mixtures (50μl) were 1.5mM $MgCl_2$; 50mM KCl; 10mM Tris-HCl, pH 8.3; 200μM each deoxynucleotide triphosphate, and contained 0.2μM of primer RT1 and RT2, template DNA (2μl), and Amplitaq polymerase as indicated. The reactions were carried out for 40 cycles using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Each cycle included a heat denaturation step at 94℃ for 1 min, followed by annealing of the primers to the template DNA at 40℃ for 2 min, and DNA chain extension with Amplitaq polymerase for 2 min at 72℃.

For the second PCR amplification, the first-PCR product (5μl) was amplified using oligonucleotide primers of KT1 and KT2, RT1 and GT2. The PCR amplification products were electrophoresed in a 1.2% agarose gel, and were stained with ethidium bromide.

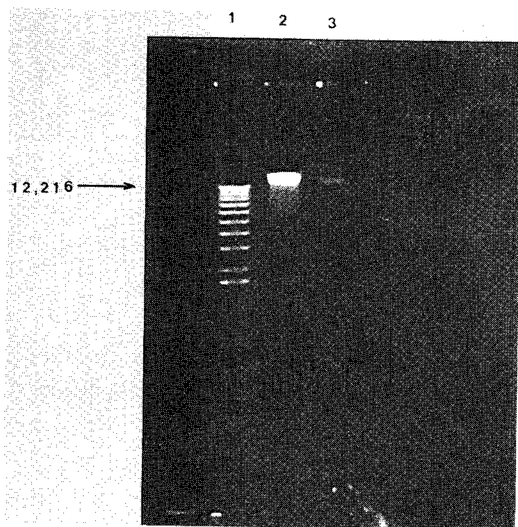

Fig. 6. Agarose gel electrophoresis of genomic DNAs of the Karp and Gilliam strains of *R. tsutsugamushi*. Lane 1, 1-kb DNA ladder as size marker: Lane 2, Karp strain; Lane 3, Gilliam strain.

RESULTS

Multiplication curves in LuMA cultures

Plaques formed by *R. tsutsugamushi* in LuMA cells after the 18th day of infection are shown in Fig. 2. The plaques were about 1-2 mm in diameter with relatively little size variation. Fig. 3. presents results obtained by infecting LuMA cells with *R. tsutsugamushi* propagated in embryonated egg yolk sac after different incubation time. At day 7, *R. tsutsugamushi* Karp strain PFU per ml was $10^{8.8}$ and Gilliam strain PFU per ml was 107.1. The PFU titer in Gilliam strain was lower than the PFU titer in Karp strain.

Cytopathic changes

The host cells were infected with *R. tsutsugamushi*, showed progressive cytopathic changes (Fig. 4 and 5). The rate and extent of cytopathic changes depended on the rickettsial concentration and the type of Rickettsiae.

PCR amplification of purified DNA

PCR was used to detect rickettsial DNA (Fig. 6) by using the gene encoding the 56Kd antigen as a target. Using this PCR, purified rickettsial DNAs from Karp and Gilliam strains of *R. tsutsugamushi* were analyzed. The predicted 408-bp DNA fragments were identified by agarose gel electrophoresis with template DNAs from Karp strains of *R. tsutsugamushi*, and 381-bp DNA fragments from Gilliam strains of *R. tsutsugamushi* (Fig. 7).

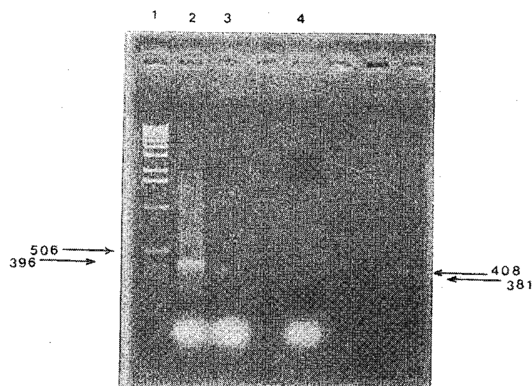

Fig. 7. Agarose gel electrophoresis of PCR amplification products. Lane 1, 1 kb DNA ladder as size marker ; Lane 2, Karp strain ; Lane 3, Gilliam strain. The numbers beside the panels are sizes in base pairs.

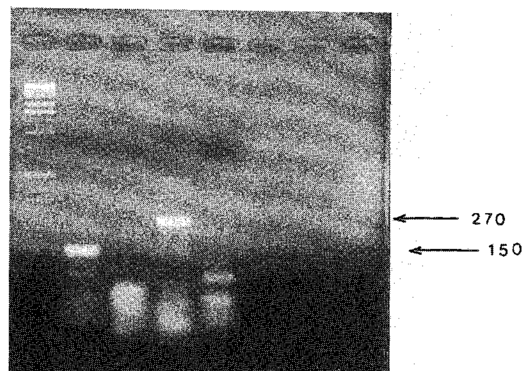

Fig. 8. Agarose gel electrophoresis of PCR amplification products by nested-PCR with serotype-specific primers for Karp (Lane 2, Lane 5) and Gilliam strains (Lane 3, Lane 4). The DNA marker used was a 1-kb DNA ladder.

Nested PCR

After the first PCR with primers RT1 and RT2, PCR amplification products were used as templates in the second PCR with strain-spec-

ific primers. The Karp and Gilliam strains were amplified by nested PCR with primer pairs KT1 and KT2, RT1 and GT2. PCR with Gilliam-specific primers was expected to yield 270-bp DNA fragment. PCR with Karp-specific primers was expected to yield 150-bp DNA fragment. With Gilliam-specific primers, the predicted 270-bp DNA fragment was identified as a band by agarose gel electrophoresis with template DNA from the Gilliam strain. However, no PCR amplified band was detected when DNAs from the Karp strain was used. Also, after PCR with strain Karp-specific primers, the predicted 150-bp DNA fragment was identified as band when template DNA from only the respective strain was used (Fig. 8). These results indicate that PCR amplification with the primers is specific for each *R. tsutsugamushi* strain.

DISCUSSION

As one of the attempts to select the adequate cell line to be used for propagation and plaquing of *R. tsusugamushi*, multiplication of the rickettsiae was followed up in LuMA cell [18]. Plaque formation was also achieved with both L-929 cells and chicken embryo (CE) cells [33,34]. Plaques in L-929 cells had its technical difficulties. Although chicken embryo cell cultures have been used for plaque titrations of rickettsiae, maintaining of a sterile intact monolayer for the incubation period required by the scrub typhus group has been very difficult. This study has clearly demonstrated that *R. tsusugamushi* will form plaques in LuMA cells [15] that are particularly suitable for the syudy of specialized aspects of rickettsial biology. Successful plaquing in LuMA cells is an important observation for future studies in vaccine development.

The nested PCR described here amplified the rickettsial DNA, and the serotypes were determined. Nested PCR [14] is a very rapid and sensitive means of detecting *R. tsutsugamushi* DNA. Application of this method to clinical specimens from acute-phase patients suggests its usefulness for diagnosis of tsutsugamushi serotypes.

SUMMARY

We selected the adequate cell line to be used for propagation and plaquing of R. *tsutsugamushi* in laboratory and identified R. *tsutsugamushi* serotype cultured in LuMA cells by nested PCR. As in this study, we concluded that.

1. LuMA cell was suitable for the study of the biology of rickettsiae-host cell interaction.
2. The plaque-forming unit (PFU) per ml of R. *tsutsugamushi* Karp strain propagated in embryonated egg yolk sacs was $10^{8.8}$ and the PFU/ml of Gilliam strain was $10^{7.1}$.
3. The rate and extent of cytopathic changes depended on the PFU titer of R. *tsutsugamushi*.
4. PCR with nested primer pairs was useful for identification of *R. tsutsugamushi* serotype cultured in human embryonic lung cells.

REFERENCES

1. Barker LF, Patt JK, Hopps HE: Titration and neutralization of *Rickettsia tsutsugamushi* in tissue culture. J Immunol 100: 825-830, 1968.
2. Barker LF, Patt JK: Production of rickettsial complement-fixing antigens in tissue culture. J Immunol 100: 821-824, 1968.
3. Bozeman FM, Elisberg BL: Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc Soc Exp Biol Med 112: 568-576, 1963.
4. Cadigan FC, Andre RG, Bolton M, Gan E, Walker JS: The effect of habitat on the prevalence of human scrub typhus in Malaysia. Transactions Royal Soc Trop Med Hyg 66: 582-587, 1972.
5. Chang, Woo H, Kang, Jae S, Lee, Woo K, Choi, Myung S, Lee, Jeung H: Serological

classification by monoclonal antibodies of *Rickettsia tsutsugamushi* isolated in Korea. J Clin Microbiol 28: 685-688, 1990.
6. Chun, Bok K, Park, Song Y, Moon, Hong M: Propagation of varicella-zoster virus in human embryonic lung cells using microcarrier. J Korean Soc Virology 24: 43-51, 1994.
7. Cohn ZA, Bozeman FM, Campbell JM, Humphries JW, Sawver TK: Study on growth of rickettsiae. J Exp Med 109: 271-292, 1959.
8. Dohany AL, Lim BL, Huxsoll DL: Vectors of scrub typhus and their hosts on a mature oil palm estate. Southeast Asian J Trop Med Pub Hlth 10: 510-513, 1979.
9. Dohany AL, Shirai A, Robinson DM, Ram S, Huxsoll DL: Identification and antigenic typing of *Rickrttsia tsutsugamushi* in naturally infected chiggers (Acarina: Trombiculidae) by direct immunofluorescence. Am J Trop Med Hyg 27 (6): 1261-1264, 1978.
10. Dohany AL: Vector transmission of scrub typhus and controll of vector mites. Malaysian J Path 1: 15-18, 1978.
11. Elisberg BL, Campbell JM, Bozeman FM: Antigenic diversity of *Rickettsia tsutsugamushi* : Epidemiology and ecologic significance. J Hyg Epidemiol Microbiol Immunol 12 : 18-25, 1968.
12. Eisemann CS, Osterman JV : Identification of strain-specific and group-reactive antigenic determinants on the Karp, Gilliam and Kato strains of *Rickrttsia tsutsugamushi*. Am J Trop Med Hyg 34(6) : 1173-1178, 1985.
13. Furuya Y, Yoshida Y, Katayama T, Kawamori F, Yamamoto S, Ohashi N, Tamura A, Kawamura A : Specific amplification of *Rickrttsia tsutsugamushi* DNA from clinical specimens by polymerase chain reaction. J Clin Microbiol 29 : 2628-2630, 1991.
14. Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A: Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nested polymerase chain reaction. J Clin Microbiol 31, 1637-1640, 1993.
15. Woo, Gyu J, Hwang, Kyu K, Chun, Bok H, Park, Song Y, Moon, Hong M : Plaque assay for varicella-zoster virus titration. J Korean Soc Virology 24: 123-128, 1994.
16. Hanson B: Identification and partial characterization of *Rickrttsia tsutsugamushi* major protein immunogens. Infect Immun 50: 603-609, 1985.
17. Hanson B: Factors influencing *Rickrttsia tsutsugamushi* infection of cultured cells. Am J Trop Med Hyg 36(3): 624-630, 1987.
18. Hwang, Kyu K, Woo, Gyu J, Chun, Bok H, Park, Song Y, Moon, Hong M : Development of a live attenuated varicella vaccine (MAV/06 strain): immunological and clinical responses in healthy persons. J Korean Soc Virology 24: 111-122, 1994.
19. Hopps HE, Jackson EB, Danauskas JX, Smadel JE.: Study on the growth of rickettsiae. J Immonol 82: 161-171, 1959.
20. lida T, Kawashima H, Kawamura A: Direct immuno fluorescence for typing of Tsutsugamushi disease rickettsia. J Immunol 95: 1129-1133, 1966.
21. Jackson EB, Danauskas JX, Smadel JE, Fuller HS, Coale MC, Bozeman FM : Occurrence of *Rickrttsia tsutsugamushi* in Korean rodents and chiggers. Am J Hyg 66: 309-320, 1957.
22. Kobayashi Y, Nagai K, Tachibana N: Purification of complement fixing antigens of *Rickettsia orientalis* by ether extraction. Am J Trop Med Hyg 18: 942-952, 1969.
23. Ng FKP, Oaks SC, Lee M, Groves MG, Lewis GE: A scanning and transmission electron microscopic examination of *Rickettsia tsutsugamushi*-infected human endothelial, MRC-5, and L-929 cells. Japan

J Med Sci Biol 38: 125-139, 1985.

24. Oaks EV, Stover CK, Rice RM: Molecular cloning and expression of Rickrttsia *tsutsugamushi* genes for two major protein antigens in *Escherichia* coli. Infect Immun 55: 1156-1162, 1987.
25. Oaks SC, Hetrick FM, Osterman JV: A plaque reduction assay for studying antigenic relationships among strains of *Rickettsia tsutsugamushi*. Am J Trop Med Hyg 19: 998-1006, 1980.
26. Ohashi N, Nashimoto H, Ikeda H, Tamura A: Cloning and sequencing of the gene (*tsg56*) encoding a type-specific antigen from *Rickrttsia tsutsugamushi*. Gene 91: 119-122, 1990.
27. Ohashi N, Tamura A, Ohta M, Hayashi K: Purification and characterization of a type-specific antigen of *Rickettsia tsutsugamushi*. Infect Immun 57: 1427-1431, 1989.
28. Shirai A, Dohany AL, Gan E, Chan TC, Huxsoll DL: Antigenic classification of *Rickettsia tsutsugamushi* isolates from small mammals trapped in developing oil palm complex in peninsular Malaysia. Japanese J Med Sci Biol 33: 231-234, 1980.
29. Stover CK, Marana DP, Dasch GA, Oaks EV: Molecular cloning and sequence analysis of the Sta58 major antigen gene of *Rickrttsia tsutsugamushi*: Sequence homology and antigenic comparison of Sta58 to the 6o-kilodalton family of stress proteins. Infect Immun 58: 1360-1368, 1990.
30. Stover CK, Marana DP, Carter JM, Roe BA, Mardis E, Oaks EV: The 56-kilodalton major protein of *Rickrttsia tsutsugamushi* : molecular cloning and sequence analysis of the sta56 gene and precise identification of a strain-specific epitope. Infect Immun 58: 2076-2084, 1990.
31. Urakami H, Tsuruhara T, Tamura A: Electron microscopic studies on intracellular multiplication of *Rickrttsia tsutsugamushi* in L cells. Microbiol Immunol 28: 1191-1201, 1984.
32. Urakami H, Tsuruhara T, Tamura A: Penetration of Rickettsia tsutsugamushi into cultured mouse fibroblasts (L cells). Microbiol Immunol 27: 251-263, 1983.
33. Wisseman CL, Edlinger EA, Waddel AD, Jones MR: Infection cycle of *Rickettsia rickettsii* in chicken embryo and L-929 cells in culture. Infect Immun 14: 1052-1064, 1976.
34. Woodman DR, Gray R, Weiss E: Improved chicken embryo cell culture plaque assay for scrub typhus rickettsiae. J Clin Microbiol 6: 639-641, 1977.