Bangl. J. Vet. Med. (2005). 3 (2): 160–165

CHARACTERIZATION OF HUMAN GRANULOCYTIC EHRLICHIOSIS IN EXPERIMENTAL INFECTED HL-60 CELLS WITH *ANAPLASMA PHAGOCYTOPHILUM* USING NESTED PCR AND *A. PHAGOCYTOPHILUM* MAJOR SURFACE PROTEIN-2 MONOCLONAL ANTIBODY

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ABSTRACT

The study was carried out to characterize the human granulocytic ehrlichiosis in experimental infected HL-60 cells with *Anaplasma phagocytophilum* using nested PCR and *A. phagocytophilum* major surface protein-2 monoclonal antibody. The nested PCR revealed only one band of 926 base pair DNA from *A. phagocytophilum* infected HL-60 cells. The western blot revealed several bands with a dominant 44-kDa. There were intense band of 100- and 160-kDa bands. The 44-kDa component was at least 10 times more abundant than the 100- and 160-kDa bands. In conclusion, the nested PCR would be a valuable tool for the characterization of human granulocytic ehrlichiosis.

Key words: Characterization, *Anaplasma phagocytophilum*, granulocyte, PCR, monoclonal antibody, human

INTRODUCTION

Tick-borne diseases represent a public health problem of growing importance. The emergence and recognition of an increasing number of new tick borne diseases in recent years highlights the significance of this zoonosis (Parola and Raoult, 2001ab; Oteo, 2001).

Ehrlichiosis comprises a group of emerging infectious tick borne diseases caused by obligate intracellular Gram-negative bacteria that infect leukocytes. Some of these have been demonstrated to be human pathogens (Chen *et al.*, 1994; Parola and Raoult, , 2001a).

The etiologic agent of human granulocytic ehrlichiosis (HGE) is closely related to *Ehrlichia phagocytophilum* and *E. equi* (Chen *et al.*, 1994). However, these species are now considered one species, and they were renamed *Anaplasma phagocytophilum* in a recent classification (Dumler *et al.*, 2001). The disease first recognized in the upper midwestern United States in 1994 (Bakken *et al.*, 1994). The disease is also widespread in Scandinavia (Tuomi, 1967) and other parts of mainland Europe (Woldehiwet and Scott, 1993) and Asia (Park *et al.*, 2003; Heo *et al.*, 2003). Patients with HGE often present relatively nonspecific symptoms that include fever, myalgia, headache, chills, lethargy, arthralgia, leukopenia, thrombocytopenia, and a mild elevation in levels of transaminases (Bakken *et al.*, 1996; Walker and Dumler, 1996). Studies of ehrlichiae in HL-60 promyelocyte cells was first reported in 1995, and several isolates have subsequently been studied from different geographic regions where HGE has been reported (Goodman *et al.*, 1996; Telford *et al.*, 1996; Rikihisa *et al.*, 1997). Thus, the studies of the isolates allow the study of the differences between individual isolates and relationship of specific molecular and antigenic components to infection. Therefore, in this study we characterized the human granulocytic ehrlichiosis in experimentally infected HL-60 cells with *Anaplasma phagocytophilum* Webster strain using nested Polymerase Chain Reaction through the amplification of 16S rRNA gene and immunoblotting through major surface protein-2 monoclonal antibody.

MATERIALS AND METHODS

Culture of HL-60 Cell

HL-60 cells (American Type Culture Collection, Manassas, Va.) were cultured in RPMI 1640 medium (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS) and 2mM L-glutamine (Invitrogen Corporation, Japan). The cells were checked every day for the concentration and morphology. The cultured HL-60 cells were stored at -20°C until used.

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***Anaplasma phagocytophilum* and HL-60 cells infection**

A. phagocytophilum Webster strain was kept with HL-60 cells for infection in RPMI 1640 medium supplemented with 5% fetal calf serum and 2mM L-glutamine (Goodman *et al.*, 1996; Asanovich *et al.*, 1997; Lin *et al.*, 2002). The cells were checked every day for the infection rate, when >80-90% of the HL-60 cells were infected, as determined by Diff-Quik staining (Baxter Scientific Products, Obetz, Ohio). The presence of intact bacteria was confirmed by Ramanowsky staining (HEMA 3, Biochemical Science, Swedesboro, NJ) and indirect fluorescent-antibody assay (IFA). The infected HL-60 cells were stored at -20°C until used.

Nested Polymerase Chain Reaction (PCR)

DNA was extracted from HL-60 cells and *A. phagocytophilum* infected HL-60 cells using the GENE√ALL™ *Tissue SV plus!* mini kit (General Biosystem, Korea). Briefly, cells ($5x10^6$) in a 1.5 ml microcentrifuge tube were centrifuged at 14,000xg for 20 sec. The supernatant was discarded as much as possible and thoroughly resuspended the cell pellet in 200 microliters of Buffer TL (Tissue Lysis Solution). Twenty microliters of Proteinase K solution (20mg/ml) was added, mixed thoroughly by vortexing and incubated for 10 min at 56°C. The tube was spin down briefly to remove any drops from inside of the lid and 400 microliters of Buffer TB (Tissue Binding Solution) added. The tube was vortexed briefly to mix thoroughly, and spin down briefly to remove any drops from inside of the lid. The mixture was applied to the spin column, centrifuged for 1 min at 600xg and replaced the collected tube with new one. Six hundred microliters of Buffer BW (Column Wash Solutin B) was added, centrifuged for 1 min at full speed and replaced the collection tube with new one. Seven hundred microliters of Buffer TW (Column Wash Solution T) was applied and centrifuged for 1 min at full speed. Discarded the flow through and reinserted the Spin Column back into the collection tube. Centrifuged at full speed for 2 min to remove residual wash buffer and placed the spin column in a fresh 1.5 ml tube. Two hundred microliters of Buffer AE (Elution solutin-10Mm TrisCl, pH 9.0, 0.5Mm EDTA) and incubated for 2 min at room temperature. Centrifuged at full speed for 1 min and DNA were stored at -20°C until used.

A nested PCR was performed with the primers designed to amplify the 16 rRNA gene of *A. phagocytophilum* (Cao *et al.*, 2000). Primers used in the nested PCR were: EE-1F (5' -TCC TGG CTC AGA ACG AAC GCT GGC GGC- 3') and EE-2R (5'-AGT CAC TGA CCC AAC CTT AAA TGG CTG- 3') for primary amplification and EE-3F (5'-GTC GAA CGG ATT ATT CTT TAT AGC TTG C- 3') and EE-4R (5' –CCC TTC CGT TAA GAA GGA TCT AAT CTC C- 3') for secondary amplification. The PCR amplifications were performed in a volume of 20 microliters in a Perkin-Elmer (Norwalk. CT) model 2400 thermal cycler. An initial three minutes denaturation at 95°C was followed by 35 cycles at 94°C for 15 seconds, 55°C for 20 seconds, and 72°C for 15 seconds, and a final extension at 72°C for 5 minutes. In nested PCR, the components and conditions were similar to those for the primary amplification, except that the primers and the primary PCR product were used as the template. A positive control a plasmid containing the rRNA gene of the HGE agent. The reaction products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using still video documentation system (Gel Doc 2000, Bio Rad, USA). To minimize contamination, DNA extraction, the reagent setup, amplification, and agarose gel electrophoresis were performed in separate room.

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE and immunoblotting were performed as described (Laemmli, 1970; Dumler *et al.*, 1995). Frozen stocks HL-60 cells and *A. phagocytophilum* infected HL-60 cells were rapidly thawed at room temperature and then centrifuged in a microcentrifuge at 13000 rpm 2 min to pellet, separately. The pellet was resuspended in SDS-PAGE sample buffer (final concentration: 32 mM TRIS-HCL, 5 mM EDTA, 13.8% 2-mercaptoethanol, 3.6% saturated bromophenol blue solution, 36% glycerol). Samples were heated at 95°C for 5 min. Samples (25 microliters each, separately) was loaded into lanes of an 18x20x15 cm polyacrylamide gel consisting of a 2.5% stacking layer and a 12.5% separating layer. Electrophoresis was continued with 100 volt until the dye front come at the end. Gels were used for electrotransfer to nitrocellulose for immunoblotting.

Immunoblotting

Proteins that were separated by SDS-PAGE were transferred to a 15x15 cm nitrocellulose paper with 50 volt for overnight (Tobwin *et al.*, 1979). After electrotransfer, the blots were carefully labeled and blocked by preincubation in 0.1 M PBS with 1% nonfat dry milk, 0.05% Tween 20 (PBSMT) for 10 min at room temperature. The immunoblots were then incubated at room temperature for 2 hours with *A. phagocytophilum* major surface protein-2 monoclonal antibody 20B4 diluted at 1:1000 in (PBSMT) for 2 hours at room temperature with rocking. Hybridoma clones producing major surface protein-2 monoclonal antibody to *A. phagocytophilum* (Webster strain) was selected by protein blotting (Park *et al.*, 2003).

The immunoblots were washed 3 times with PBS with 0.05% Tween 20 for 5 min each wash and then incubated for 2 hours with the anti-mouse IgG (Kierkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in PBSMT. After another wash bound antibodies were visualized with BCIP/NBT (5-bromo-4 chloro-3-indolyl phosphate at 170 microgram/mL with nitroblue tetrazolium at 330 microgram/mL in 100 mM TRIS-HCL, pH 9.5, 100 mM Nacl, and 5 mM Mgcl2 alkaline phosphate substrate buffer; Sigma, St. Louis).The immunoblots were ringed with deionized water and air-dried for 15 min. The molecular size of the visualized bands were determined by comparison with the molecular size protein standards (prestained high molecular weight protein standard, GIBCO-BRL, Gaithersburg, MD).

RESULTS AND DISCUSSION

The result of nested PCR has been shown in Figure 1. It revealed that there was only one band of 926 base pair DNA from *A. phagocytophilum* infected HL-60 cells. There were no bands from the normal HL-60 cells. The result of western blot has been shown in Figure 2. It reveals several bands with a dominant 44-kDa. There were intense band of 100- and 160-kDa bands. The 44-kDa component was at least 10 times more abundant than the 100- and 160-kDa bands.

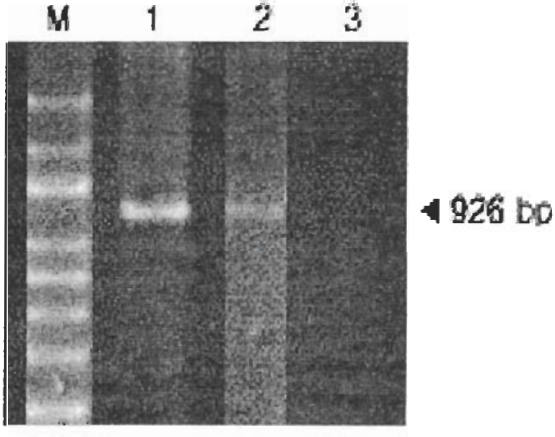

Fig. 1. Result of Nested PCR. M, 100-bp size marker (iNtRON Biotechnology, Korea); 1, Amplicon from positive control of 926-bp *A. phagocytophilum* 16S rRNA; 2, amplicon of 926-bp *A. phagocytophilum* 16S rRNA from *A. phagocytophilum* infected HL-60 cells; 3, no amplicon from normal HL-60 cells.

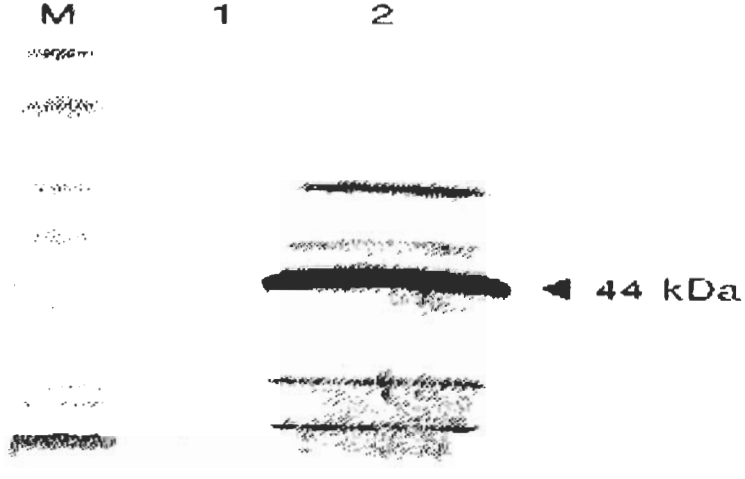

Fig. 2. Result of western blot assay using *A. phagocytophilum* major surface protein-2 monoclonal antibody. M, protein size marker (Invitrogen, USA); 1, normal HL-60 cells; 2, *A. phagocytophilum* infected HL-60 cells showing the distinct band of 44 kDa.

The diagnosis of HGE depends on evaluation of clinical, laboratory, and epidemiological data. *A. phagocytophilum* and *E. chaffeensis* infections are characterized by presence of intracytoplasmic inclusions called morulae within leukocytes of human or animal peripheral blood smears. However, since it is difficult to detect *A. phagocytophilum* and *E. chaffeensis*, examination of blood smears for morulae is not a sensitive approach for laboratory diagnosis.

To date, no cultures of the HGE agent have been performed in Europe and Asia. Cultures are positive in 34.8% of cases, independent of the incubation period or the characteristics of initial symptoms (Horowitz *et al.*, 1998). The success in finding morulae varies in accordance with the experience of the microscopist and the duration of illness (Bakken *et al.*, 2000).

Serology may give false-positive results (Lascola *et al.*, 1997). Cross-reactivity between the HGE agent and E. chaffeensis has been described in 30-53.8% of patients with confirmed and probable HGE (Comer *et al.*, 1999; Wallace *et al.*, 1998). Cross-reactivity to the HGE agent and other rickettsial agents (*Coxiella burnrtti*, *Rickettsia rickettsii* and *Rickettsia typhi*) has also been described (Comer *et al.*, 1999; Guerrero *et al.*, 2001).

Although the indirect fluorescent-antibody assay (IFA) is the most sensitive method, it can give inconsistent results because of antigenic diversity and various technical factors among different laboratories. For example, use of the MRK strain of *A. phagocytophilum* (*Ehrlichia equi*) as the antigen for IFA revealed a high degree of variability even if tests were performed in the same laboratory (Asanovich *et al.*, 1997). Western blot assays are generally thought to provide more detailed information than IFA about the specific reactive antigens and our laboratory already reported the HGE agent using the western blot and IFA (Park *et al.*, 2003; Heo *et al.*, 2003) in Korean patients.

Methods based on PCR gene amplification could be useful, sensitive and rapid for the detection and identification of tick-borne pathogens (Parola and Raoult, 2001b; Inokuma *et al.*, 2001). Several PCR-based molecular assays are available for the detection of the *A. phagocytophilum* and different types of primer can be employed e.g. ge2, ge9f, ge10r (Parola and Raoult, 2001b; Lotric-Furlan *et al.*, 1998; Tylewska-Wierzbanowska *et al.*, 2001; Bjoersdorff *et al.*, 1999; Inokuma *et al.*, 2001; Garcia-Perez *et al.*, 2000).

The *A. phagocytophilum* infected HL-60 cells and normal HL-60 cells were characterized by the nested PCR and additionally the western blot using the *A. phagocytophilum* major surface protein-2 monoclonal antibody was also applied. The western blot revealed several bands with a dominant 44-kDa and the nested PCR assay identified 926 base pair DNA in PCR products

There are a large number of factors involved in optimizing a PCR assay that has a low limit of detection and is also specific and reproducible. Some of these variables include the PCR kit and the enzyme used; Mg^{2+}, template, and primer concentrations; optimization factors specific to the assay, such as the annealing temperature and number of cycles; the method used for extraction; and the thermal cycler used amplification. While we attempt to control for many of these factors, the large number of potential variables makes it difficult to predict the performance of a given assay in an outside laboratory or whether significant improvement in the performance of single assays could be achieved by optimizing assay contitions. Each laboratory should determine the efficacy of any PCR assay within their local environment.

In this study, we standardized the nested PCR. The nested PCR assay described in the present study has several advantages over the serological method to identify *A. phagocytophilum*. A major advantage was the speed with which the assay can be performed, i.e. within a day. The serological method required at least several days. Moreover, the nested PCR is typically more sensitive than the direct PCR. In conclusion, the nested PCR would be a valuable tool for the characterization of human granulocytic ehrlichiosis.

ACKNOWLEDGEMENTS

Dr Md. Siddiqur Rahman was supported in part by funding of the Korea Science and Engineering Foundation (KOSEF), South Korea as postdoctoral fellowship (March, 2004-March, 2005).

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