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Biochemical and molecular characterization of a strain KA/S2 of *Acanthamoeba castellanii* isolated from Korean soil

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Abstract: A strain, KA/S2, isolated from Korean soil and morphologically assigned to *Acanthamoeba castellanii*, was characterized by isoenzyme analysis, and total proteins profile, and mitochondrial (Mt) DNA restriction fragment length polymorphism (RFLP), and compared with four reference strains assigned to the species (the authenitic Castellani, Neff, Ma, and Chang strains). It was found that four isoenzyme, total proteins, and Mt DNA RFLP patterns by eight restriction endonucleases of the strain KA/S2 were identical with those of the Neff strain, isolated from soil of California, USA. The Chang strain was unique in its morphology and total protein patterns. Interstrain polymorphisms of isoenzyme profiles and Mt DNA RFLP patterns were observed among the Castellani, Neff, Ma, and Chang strains. Mt DNA RFLP was confirmed to be highly appropriate for the strain characterization and identification of *Acanthamoeba* spp.

Key words: *Acanthamoeba castellanii*, KA/S2, Neff strain, isoenzymes IEF, Mt DNA RFLP, strain characterization and identification.

INTRODUCTION

Acanthamoeba castellanii (Douglas 1930) Volkonsky, 1931 has been known to be one of the most frequently occurring species in clinical amoebic keratitis (Kilvington *et al.*, 1991). Among several strains of *A. castellanii*, the Neff strain has been studied most extensively (Neff, 1957; MacKay & Doolittle, 1981; Gunderson & Sogin, 1986; Byers *et al.*, 1990; Kilvington *et al.*, 1991; Vodkin *et al.*, 1992; Burger *et al.*, 1995) whereas the other strains including the authentic strain have yet to be studied. Recently, Burger *et al.* (1995) determined the complete primary sequence of mitochondrial (Mt) genome of *A. castellanii* Neff strain. However, as Kong *et al.* (1995) indicated, interstrain diversity exists among the strains assigned to *A. castellanii*. Therefore, the results obtained from the Neff strain may not be representative of all *A. castellanii* strains. Kong *et al.* (1995) suggested that isoenzyme and mitochondrial (Mt) DNA restriction fragment length polymorphism (RFLP) analyses can be appropriate tools for strain identification and characterization of *Acanthamoeba* spp.

The present authors have isolated *Acanthamoeba* spp. from soil, cooling tower

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water, and contact lens cases. Among them, a soil isolate, KA/S2, was morphologically assigned to *A. castellanii*. In the present study, the strain KA/S2 was characterized and identified by comparing isoenzyme profile, total protein pattern and Mt DNA digestion phenotype with those of four reference strains, the authentic Castellani, Neff, Ma, and Chang, of different sources (Table 1).

MATERIALS AND METHODS

Isolation and axenic culture

Acanthamoeba strain KA/S2 was isolated from Korean soil and axenically cultured by the methods described by Kong *et al.* (1995) and assigned morphologically to *A. castellanii* according to the keys described by De Jonckheere (1987) and Page (1988).

Reference strains of *A. castellanii*

Four strains of *A. castellanii* (ATCC # 30010 Neff strain, # 30011 the authentic Castellani strain, # 30898 Chang strain and # 50370 Ma strain) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and used as reference strains (Table 1).

Isoenzyme isoelectric focusing (IEF)

Isoenzyme IEF was performed as previously described by Kong *et al.* (1995). Briefly, the trophic amoebae harvested at the end of the logarithmic growth phase were homogenized by freezing at −70°C and thawing at 37°C. The lysate was run on precast vertical IEF gel, pH 3~10 and 1mm thick (Novex Experimental Technology, USA). After focusing, four kinds of isoenzymes, acid phosphatase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, and, leucine amino peptidase were developed by conditions summarized in Table 2. The gel in the developing solution was incubated in darkness at 37°C with gentle shaking. The reaction was stopped and the gel was fixed by 7.5% acetic acid solution. The gel was then differentiate in 10% acetic acid and 25 % ethanol until a clear background was obtained.

Total proteins IEF

The same amoeba lysate as used in the isoenzyme analysis was run on precast vertical IEF gel, pH 3-7 and 1mm thick (Novex Experimental Technology, USA). Instead of enzyme development, the gel was stained with silver nitrate.

Mitochondrial (Mt) DNA RFLP analysis

Mt DNA of *A. castellanii* KA/S2 and reference strains was extracted by the method described by Yagita and Endo (1990). Eight restriction endonucleases (*Bgl* II, *Hpa* I, *Sca* I & *Xba* I obtained from Promega, USA; *Sst* I from Gibco BRL, USA; *Cla* I, *Eco*R I, & *Sal* I from Kosco, Korea) were used for the Mt DNA RFLP analysis. The DNA was digested with 1~6 units of restriction enzyme at 37°C for 2 hours (sometimes overnight) in 20 μl reaction volume with the buffers specified for each restriction enzyme. Digested DNA was electrophoresed in 0.7% agarose gel at 4 V/cm for 1~2 hours and stained with ethidium bromide. The Mt DNA RFLP patterns of five strains were observed and photographed under UV transilluminator. The *Hind* III digested λ phage was used as a size marker.

Statistical analysis of the difference in size and arm number of the cyst.

One way ANOVA was applied to analyse the significance of the difference in size and arm number of cyst among five strains.

Table 1. Morphology of *Acanthamoeba castellanii* KA/S2 and the reference strains

	Strain	Source	Cyst diameter (μm)		Number of arms		References
			mean	range	mean	range	
1	KA/S2	soil, Korea	16.7	14~20	7.4	6~9	—
2	Castellani	yeast culture	15.6	13~18	7.0	5~10	Douglas (1930)
3	Neff	soil, USA	16.2	13~20	7.0	6~8	Neff (1957)
4	Ma	keratitis	15.7	13~18	4.6	4~7	Ma (1981)
5	Chang	fresh-water	18.7	15~22	4.8	4~6	Ryers (1990)

Table 2. Development conditions of enzymes tested (final concentration/100 ml)

Enzyme	Substrate	Coenzyme	Linking enzyme	Other reagents	Buffer	Staining time
AcP[a] (EC[b]3.1.3.1)	β-naphthyl acid phosphate (Na) 100 mg			Black K salt 100 mg	0.05 M Acetate pH 5.0	40 min
G6PD[c] (EC1.1.1.49)	D-Glucose 6-phosphate (Na) 45 mg	NADP[d] 45 mg		MTT[e] 16 mg PMS[f] 8 mg $MgCl_2 6H_2O$ 170 mg	0.1 M Tris-HCl pH 7.4	40 min
GPI[g] (EC5.3.1.9)	D-Fluctose 6-phosphate 80 mg	NADP 20 mg	G6PD 50 units	MTT 16 mg PMS 6 mg $MgCl_2 6H_2O$ 150 mg	0.1 M Tris-HCl pH 8.0	1 h
LAP[h] (EC3.4.1.1)	L-leucine β-naphthyl amide 40 mg	NAD[i] 32 mg		Black K salt 50 mg	0.2 M Tris-maleate pH 6.0	1 h

[a]AcP, acid phosphatase; [b]EC, enzyme commission number; [c]G6PD, glucose-6-phosphate dehydrogenase; [d]NADP, nicotinamide adenine dinucleotide phosphate; [e]MTT, 3-(4,5-dimethyl tiazolyl)-2, 5-diphenyl tetrazolium bromide; [f]PMS, phenazine methosulfate; [g]GPI, glucose phosphate isomerase; [h]LAP, leucine amino peptidase; [i]NAD, nicotinamide adenine dinucleotide.

RESULTS

Morphology

Table 1 shows cyst morphology of KA/S2 and four reference strains of *Acanthamoeba castellanii* analysed in this study. The cyst morphology of the strain KA/S2 was the most similar to that of the Neff strain (Fig. 1). The cyst of the Chang strain was significantly larger than those of the other strains. The Ma and Chang strains were significantly less in number of arms than the remaining three strains.

Total proteins

Total protein patterns of the strains of *Acanthamoeba* produced by IEF in pH 3.0 to 7.0 are shown in Fig. 3. The majority of the bands were condensed between pH 4.0~6.5 regardless of the strain, and showed similarity in patterns. Above the level of pH 6.5, a few bands were shown. The banding pattern of strain KA/S2 (lane 1) was found to be identical with that of the Neff strain (lane 3). The banding pattern of the Chang strain (lane 5) was unique.

Isoenzyme profiles

The zymograms for acid phosphatase (AcP), glucose 6-phosphate dehydrogenase (G6PD), glucose phosphate isomerase (GPI), and leucine amino peptidase (LAP) of five strains are shown in Fig. 2. The bands for AcP were widely distributed at pI range from approximately 4.5 to 7.5. The zymograms of the strains for GPI and LAP were very similar to one another. No bands were observed for G6PD from the Catellani and Ma strains, and

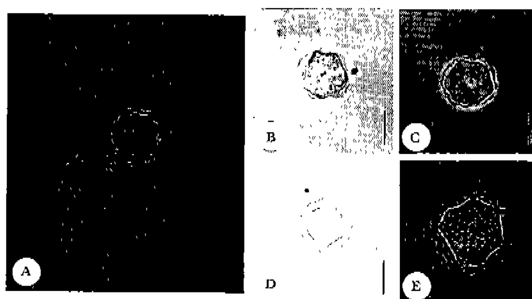

Fig. 1. Photomicrographs of the cysts of *Acanthamoeba castellanii*. **A.** KA/S2 strain; **B.** Castellani strain; **C.** Neff strain; **D.** Ma strain; **E.** Chang strain. Bars indicate 10 μm.

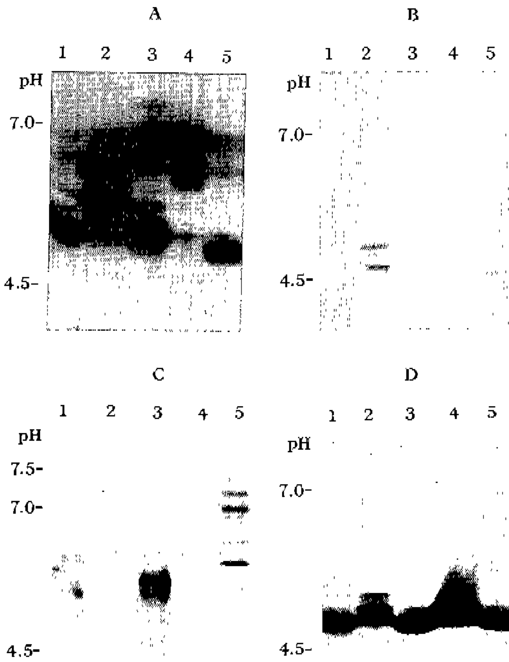

Fig. 2. Zymograms for isoenzymes of KA/S2 and reference strains of *A. castellanii* separated by polyacrylamide gel isoelectric focusing in pH gradient 3~10. **A.** Acid phosphatase; **B.** Leucine amino peptidase; **C.** Glucose-6-phosphate dehydrogenase; **D.** Glucose phosphate Isomerase. Numbers above lanes refer to the strains listed in Table 2.

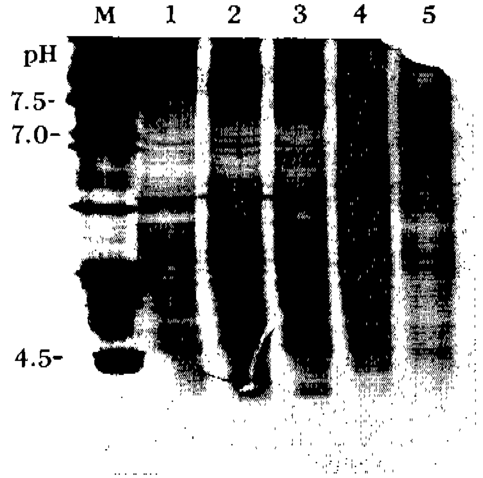

Fig. 3. Total proteins of *Acanthamoeba* separated by polyacrylamide gel isoelectric focusing in pH gradient 3-7. Numbers above lanes refer to the strains listed in Table 2.

for LAP from Ma strain. The zymograms of strain KA/S2 for four isoenzymes tested were identical to those of the Neff strain.

Mitochondrial (Mt) DNA RFLP patterns

Fig. 4 shows digestion phenotypes of KA/S2 and four reference strains of *A. castellanii* produced by eight restriction endonucleases tested. The digestion phenotypes were very different from strain to strain, except for strains KA/S2 and Neff, both of which showed the identical RFLP pattern.

DISCUSSION

Strain KA/S2, isolated from Korean soil and assigned morphologically to *Acanthamoeba castellanii*, was found to be very closely related, if not identical, with the Neff strain of the species, based on mitochondrial (Mt) DNA RFLP patterns and isoenzyme profiles. Considering the profound interstrain polymorphisms of isoenzymes and Mt DNA RFLP patterns among the strains of *Acanthamoeba* spp. (Bogler *et al.*, 1983; Byers *et al.*, 1983; McLaughlin *et al.*, 1988; Yagita and Endo, 1990; Kilvington *et al.*, 1991; Yagita, 1993; Gautom *et al.*, 1994; Kong *et al.*, 1995), strain KA/S2 seems to be the same as the Neff strain, although limited number of restriction endonucleases was applied and a few isoenzymes were studied. It is very interesting that the digestion phenotype of a Korean soil isolate was the same as that of the Neff strain, originally isolated from California soil (Neff, 1957).

The reference strains used in the present study have different characteristics. The authentic Castellani strain, originated from a yeast culture (Douglas, 1930), is the type strain for *A. castellanii*. The Castellani strain was originally known to be nonpathogenic. However, the strains isolated from corneas of keratitis patients in Japan and England were found to have the same Mt DNA digestion phenotype as that of the Castellani strain (Yagita and Endo, 1990; Gautom *et al.*, 1994). Considering fast evolution of organelle DNA compared with nuclear DNA (Ferris *et al.*, 1981), it could be speculated that strains showing the same Mt DNA digestion phenotype

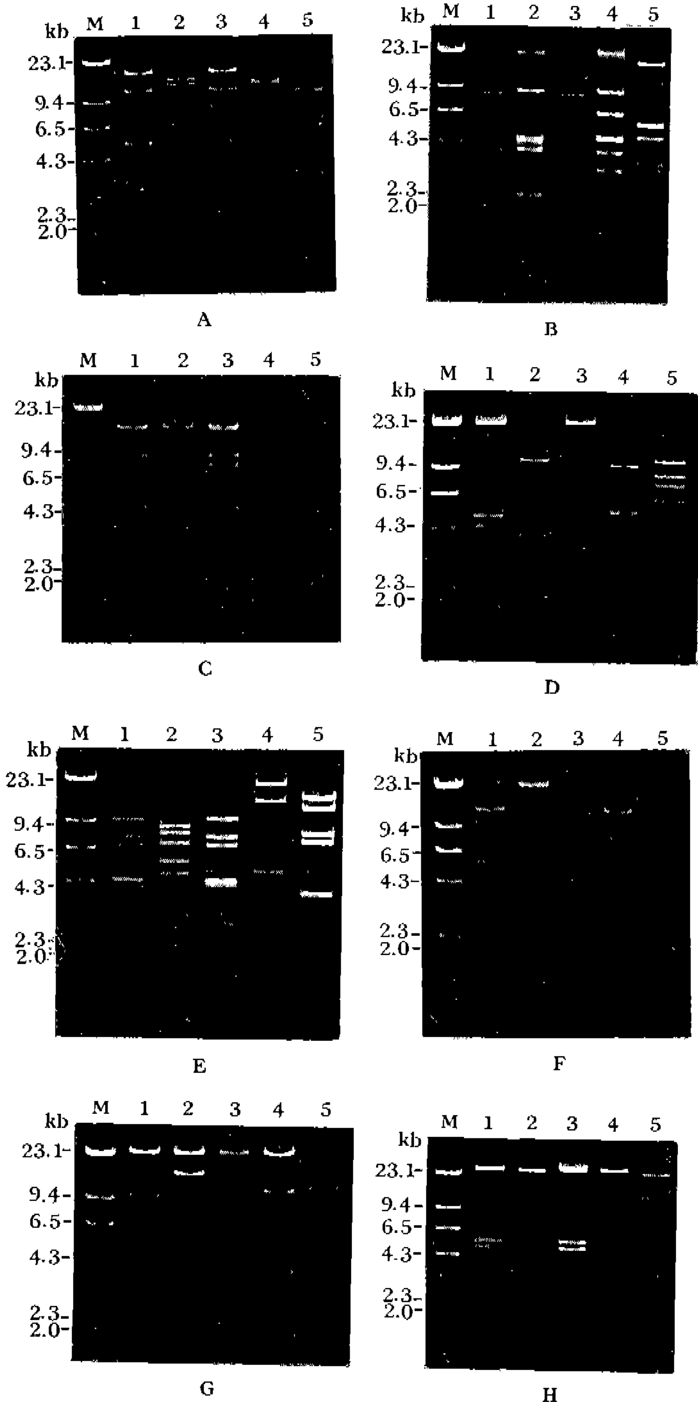

Fig. 4. Agarose gel electrophoretic fingerprints of mitochondrial DNA from KA/S2 and reference strains of *A. castellanii*. **A.** *Eco*R I digests; **B.** *Hpa* I digests; **C.** *Bgl* II digests; **D.** *Sca* I digests; **E.** *Cla* I digests; **F.** *Xba* I digests; **G.** *Sst* I digests; **H.** *Sal* I digests. Numbers above lanes refer to the strains listed in Table 2. Size marker is *Hind* III digested λ phage DNA (M).

have similar , if not the same, nuclear genes. As a matter of fact, the authors confirmed that strains showing the same Mt DNA RFLP patterns (Neff & KA/S2; Castellani & CCAP 1501/2g; Jones & Ap) revealed identical riboprints (Chung and Kong, unpublished). As indicated by Byers *et al.* (1983), pathogenicity of the Castellani strain has to be re-evaluated although the genes involved in pathogeneity are probably encoded on chromosomes, but not on mitochondiral genome.

The Neff strain, the most intensively studied one (Byers *et al.*, 1990; Kilvington *et al.*, 1991; Vodkin *et al.*, 1992; Burger *et al.*, 1995), also has yet to be evaluated for pathogeneity. Both the Ma and Chang strains are known to be highly pathogenic. The Ma strain was originated from infected human cornea (Ma *et al.*, 1981), while the Chang strain was isolated from freshwater, USA (Chang, unpublished). Among the reference strains used in the present study, the Chang strain was unique in morphology and total proteins pattern. More studies are needed to assure taxonomy of the strain.

As a matter of fact, the taxonomic examination is urgently needed especially among the strains assigned to either *A. castellanii* or *A. polyphaga*. Yagita and Endo (1990) reported that some isolates from contact lens containers and soil in Japan which had been morphologically assigned to *A. polyphaga*, showed the same Mt DNA RFLP patterns as those of the Ma strain of *A. castellanii*. *A. polyphaga* was separated from *A. castellanii* by Page (1967) mainly based on the differences of cyst morphology. However, the cyst morphological features such as diameter and number of arms are regarded unstable. Therefore, comparative biochemical and moelcular biological studies between both species are needed to evaluate the validity of taxonomy of *A. polyphaga*.

The present authors analyzed riboprints of *Acanthamoeba* strains assigned to either *A. castellanii* or *A. polyphaga* to evaluate the taxonomic validity of *A. polyphaga* (Chung and Kong, unpublished data). Based on the limited data we got, *A. polyphaga* was recognized as a junior synonym of *A. castellanii*. The authors carried out co-cultivation experiments in which cysts of both *A. castellanii* and *A. polyphaga* were mixed and cultivated before clonal selection and identification using Mt DNA RFLP. Some cysts regarded as *A. polyphaga* based on morphology revealed Mt DNA digestion phenotype of *A. castellanii*, and *vice versa*.

In the present study, the authors reconfirmed the interstrain diversity of Mt DNA RFLP pattern (Byers *et al.*, 1983; Yagita and Endo, 1990) and isoenzyme zymograms among the strains assigned to *A. castellanii*. These results support Kong *et al.* (1995) who suggested limited use of the methods, Mt DNA RFLP and isoenzyme IEF, for strain identification, characterization and differentiation rather than for taxonomic classification of *Acanthamoeba* spp.

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=초록=

카스텔란니가시아메바(*Acanthamoeba castellanii*) 한국 토양분리주 KA/S2의 생화학적 및 분자생물학적 특성

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경북대학교 의과대학 기생충학교실[1] 및 동아대학교 의과대학 기생충학교실[2]

형태적으로 *Acanthamoeba castellanii*로 동정된 한국토양분리주 KA/S2의 일부 특성을 파악하여 *A. castellanii*로 알려진 4가지 reference 주(Castellani, Neff, Ma 및 Chang주) 와 비교하였다. KA/S2주의 mitochondria(Mt) DNA RFLP와 isoelectric focusing(IEF)로 분석한 동위효소 양상은 California 토양에서 분리된 Neff주의 그것과 동일하였으나 그 외의 주들 사이에는 심한 다양성이 관찰되었다. Chang주의 형태 및 전 단백질 양상은 다른 주에 비해 독특하였다. *Acanthamoeba* spp. 분리주의 동정 및 특성 파악에 Mt DNA RFLP 분석이 매우 유용함을 확인하였다. *Acanthamoeba castellanii*의 우리발 학명을 카스텔라니가시아메바로 제안한다.

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故 崔東翊 教授를 추모하며

(1930. 1. 4-1996. 1. 17)

"경북의대와 기생충학교실의 무궁한 발전을 기원한다" 하시며 맺으신 최동익 선생님의 고별강연이 바로 며칠 전 같은데 오늘 이렇게 선생님을 추모하는 글을 써야 하니 제자 중 한 사람으로서 비통하고 황망하기 그지 없습니다.

돌이켜 보면 선생님께서는 1956년 경북대학교 의과대학을 졸업하시고 은사들의 권고에 따라 모교에 재직하시면서 학문의 길을 계속 하시기로 뜻을 세우고 의학의 많은 분야 중 기생충학을 선택하셔서 35년의 세월을 국민보건 향상, 학문의 발전, 그리고 후학의 지도에 몸 바치셨습니다. 그 당시 우리나라는 기생충병이 만연하여 전 국민이 한 가지 이상의 기생충으로 감염되어 있는 실정이었으나 기생충학은 의학계의 '고아'처럼 버려져 있었습니다. 선생님께서는 남들이 주목하지 않는 분야를 선택하여 학문의 뜻을 같이하는 선배·동료 학자들과 함께 국민보건의 향상에 괄목할 만한 성과를 이루셨습니다.

선생님께서는 1958년 간흡충의 피부반응이라는 논문을 시작으로 1995년 정년을 맞으시기까지 140여 편의 연구논문을 국내외의 전문학술지에 발표하셨고 국내외 학술대회에 빠짐없이 참가하셨습니다. 그중에서 특히 간흡충에 대한 역학적 조사와 간흡충에 대한 면역반응은 선생님께서 평생을 바쳐 연구하신 분야였습니다. 선생님의 학문에 대한 뜨거운 열정은 대한기생충학회 학술상, 보건사회부 장관 표창, 경북의사회 학술상, 유한의학 저작상 등을 수상하신 것으로도 입증이 되었습니다. 선생님께서는 학문 연구와 후학 지도를 위해 선진학문의 도입에도 남달리 관심을 가졌습니다. 일찌기 국제원자력기구(IAEA) 장학생, 세계보건기구(WHO) 장학생으로 일본 원자력연구소와 예방위생연구소에서 연구활동을 하셨고, 미국의 뉴욕주립대학에서 교환교수로 활약을 하셨습니다. 선생님께서는 회갑의 연세에도 외국대학의 Workshop에 참가하시는 열의를 보여 젊은 후학들에게 귀감이 되기도 하셨습니다.

선생님께서는 1971년 경북의대에 기생충학을 창설하셨습니다. 학부의 기생충학 강좌를 통해 많은 학생들이 잊지 못할 열강을 하셨고, 대학원 기생충학 전공주임으로 계시면서 30명의 박사와 66명의 석사를 지도하셨습니다. 특히 제자 중 주종윤 교수, 옥미선 교수를 계명의대와 고신의대에 각각 기생충학 주임교수로 보내셨고, 정동일 교수가 현재 경북의대 기생충학교실을 이어받도록 하여 영남의 기생충학을 든든하게 뒷받침하게 하셨습니다. 선생님께서는 모교인 경북의대 뿐만 아니라 영남의 각 의과대학과 충남대학에까지 원거리 출강을 마다 하지 않으시고 교육에 힘쓰신 바도 있습니다. 뿐만 아니라 선생님께서는 대한기생충학회 회장과 기생충학잡지 발행인으로 학회와 기생충학잡지의 수준을 세계적인 수준으로 끌어 올리는 데 큰 이바지를 하셨습니다. 교육자로서, 학자로서, 의료인으로서, 사회봉사자로서의 선생님의 공적이 너무도 뚜렷하여 선생님께서는 국민훈장 모란장을 수여 받으시기도 하셨습니다.

선생님의 가르침을 더 받아야 할 부분들이 아직도 너무나 많은데 선생님께서는 이미 멀리 떠나가셨습니다. 그러나 선생님을 잃은 슬픔에만 잠겨 있는 것도 선생님의 가르침에 어긋나는 것이라 여겨집니다. 평소의 가르침을 가슴에 지니고, 제자로서, 후학으로서 선생님의 뜻을 이어 나감에 게으름이 없도록 스스로 다짐하고 있습니다.

삼가 선생님의 명복을 비옵니다.

1996년 2월

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