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EMERGENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN LAOS

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Abstract: *Staphylococcus aureus* isolated in Laos (People's Democratic Republic of Lao=Lao PDR) was examined for the drug sensitivities and coagulase type. A total of 114 strains examined consisted of 32 isolated in 1995 and 47 isolated in 1996 from the infection focus, and 35 isolated from nasal mucosa of non-infected patients and nurses working in the wards. One strain with coagulase type IV was regarded as methicillin-resistant *S. aureus* (MRSA) depending on the minimum inhibitory concentration of the drugs as >100, 100, 100, >100, and 25 μ g/m*l* of methicillin, ampicillin, tetracycline, erythromycin, and cefdinir, respectively. The *mecA* gene was detected in this strain but not in the others. This is the first report of MRSA from Lao PDR.

INTRODUCTION

Although methicillin-resistant *S. aureus* (MRSA) was detected soon after the introduction of methicillin in 1960 (Barrett *et al.*, 1968), a marked rise in the frequency was seen in 1980s. In Japan, increasing of its isolation coincided with increasing use of third-generation cephem antibiotics. Since the late 1980s, more than 50% of *S. aureus* isolates at the sophisticated hospitals in Japan has been resistant to methicillin. Therefore, the careful use of these antibiotics is now recommended. In contrast, MRSA is rarely isolated in communities where antibiotic use is restricted. In People's Democratic Republic of Lao (Lao PDR), all *S. aureus* isolated at Mahosot Hospital (the most sophisticated hospital in Lao PDR) in 1993 were sensitive to methicillin (Higa *et al.*, 1994). However, urbanization of Lao PDR, especially Vientiane the Capital of Lao PDR, has been rapidly advanced. And antibiotics are more available in the market. In these situation, the emergence of MRSA should be carefully monitored. In this communication, we described the drug sensitivities and coagulase typing of *S. aureus* isolated at Mahosot Hospital in 1995 and 1996.

MATERIALS AND METHODS

Bacterial strains: *S. aureus* isolated at Mahosot Hospital, Vientiane, Lao PDR was used. The organisms included 79 strains isolated from various clinical specimens in 1995 and 1996, and 32 strains isolated from the nasal mucosa of the staff nurse and in-patients without infectious disease in 1996.

Coagulase typing: Antigenic type of coagulase produced by the isolates was examined by a neutralization test using the Coagulase Typing Immune Sera Kit (Denkaseiken Co. Tokyo). Coagulation inhibition by adding antisera was examined using plastic microdilution plates (Tajima *et al.*, 1992).

Drug sensitivity test: The sensitivities of the isolates against methicillin (DMPPC, Banyu), ampicillin (ABPC, Meiji), tetracycline (TC, Wako), erythromycin (EM, Dainihon), and cefdinir (CFDN, Fujisawa) were examined by using the plate dilution method, and the drug activities against the organisms were expressed by minimum inhibitory concentrations (MICs) of the drug. Twofold dilution series of each antibiotics in heart infusion agar (HIA) were prepared with the drug concentration ranging from 100 to 0.2 μg/m*l*. Cultures of the isolates in heart infusion broth at 37°C for 6 hr were diluted 1 to 10 with normal saline solution (ca. 10^7/m*l*) and were inoculated on the drug-containing HIA plates and drug-free control plates by using Microplanter (Sakuma Co. model MITP # 00257). MICs of each drug were evaluated after 24 hr incubation at 37°C. For the sensitivities to methicillin, the concentration of NaCl in

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drug-containing HIA was adjusted to 4%, and the culture was carried out at 30°C for 24 hr.

Detection of *mecA* gene: *mecA* was detected by using PCR analysis. The 24-hour cultured colonies on nutrient agar plates were suspended in distilled water and then boiled for 20 min, which was regarded as the samples for DNA of interest. The PCR primers used for detecting *mecA* were 5'-GAACCTCTGCTCAACAAGTT-3' and 5'-GGATTTGCCAATTAAGTTTG-3' as described by Song *et al.* (1987) and re-examined by Muraki *et al.* (1993). These were designed to amplify a 630 bp fragment derived from a portion of the *mecA* gene encoding PBP2' which has been shown to be the entity of MRSA.

RESULTS

Coagulase type: The distribution of coagulase type varied, but type V was predominant. In 1996, 63.8% of the isolates from infection sites revealed type V but only 28.6% from the nasal mucosa of non-infective individuals. Type II strains were found in 4.3% of the isolates from the infection, whereas 25.7% from the nasal mucosa of non-infected individuals.

Drug sensitivities: Generally, the isolates showed good susceptibilities against the drugs examined. A few strains were highly resistant to erythromycin. The MIC of methicillin was 6.25 μg/m*l* or less against almost all isolates, the growth of 3 isolates were inhibited at 12.5 μg/m*l* or more, and 1 of them was resistant to methicillin at 100 μg/m*l*. MICs of the other drugs against the 2 isolates were 0.8 and 3.13 μg/m*l* of ABPC, ≦0.2 and 0.8 μg/m*l* of CFDN, ≦0.2 and 0.4 μg/m*l* of TC, and >100 and ≦0.2 μg/m*l* of EM, respectively. MICs of the one isolate resistant to DMPPC at 100 μg/m*l* were 100 μg/m*l* of ABPC, 25 μg/m*l* of CFDN, 100 μg/m*l* of TC, and >100 μg/m*l* of EM. Coagulase type of this strain was type IV. These results are summarized in Tables 1, 2, 3.

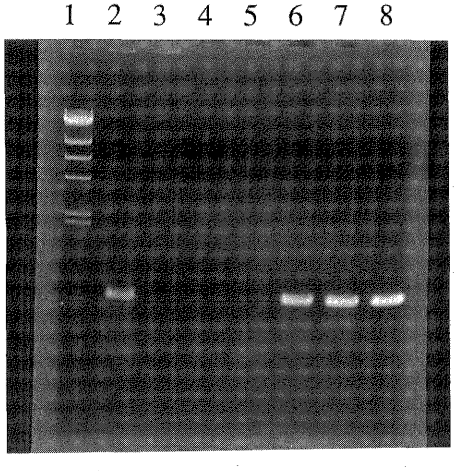

Figure 1 630 bp fragment of *mecA* gene detected as the PCR product. Lanes: 1=molecular marker, 2=LC13 (>100 μg/m*l*, MRSA), 3=LC18 (12.5 μg/m*l*), 4=95P1 (6.25 μg/m*l*), 5=96P5 (12.5 μg/m*l*), 6=R1 (>100 μg/m*l*), 7=R4 (100 μg/m*l*), 8=R12 (>100 μg/m*l*). MICs of methicillin to each strain were indicated in parenthesis. Lanes 2 to 5 are of Lao strains and lanes 6 to 8 are of Japanese strains. Only one Lao strain (LC13) of 114 examined was positive for *mecA*.

Detection of *mecA* gene: All isolates except one were negative for *mecA*. Only one strain, against which the MIC of methicillin was >100 μg/m*l*, possessed the gene (Fig. 1).

Table 1 Drug sensitivities and coagulase type

MIC (μg/m*l*)	DMPPC	ABPC	TC	EM	CFDN
≦0.2	0	6	9	27	11
0.39	0	0	0	2	16
0.78	0	4	0	0	4
1.56	1	2	0	0	0
3.13	19	12	0	0	0
6.25	12	6	7	0	0
12.5	0	2	8	0	0
25	0	0	8	0	0
50	0	0	0	0	1
100	0	0	0	0	0
>100	0	0	0	3	0

coagulase type	I	II	III	IV	V	VI	VII	VIII	UT
No. of strains	0	7	1	1	16	1	5	0	1

32 isolates from infection focus (1995)

Table 2 Drug sensitivities and coagulase type

MIC(μg/m*l*)	DMPPC	ABPC	TC	EM	CFDN
≦0.2	0	1	24	16	32
0.39	0	0	1	1	14
0.78	0	4	0	23	1
1.56	4	31	0	6	0
3.13	23	8	0	0	0
6.25	19	0	0	0	0
12.5	1	0	5	0	0
25	0	2	10	1	0
50	0	0	7	0	1
100	0	1	0	0	0
>100	0	0	0	3	0

coagulase type	I	II	III	IV	V	VI	VII	VIII	UT
No. of strains	0	2	4	2	30	0	3	1	5

47 isolates from infection focus (1996)

Table 3 Drug sensitivities and coagulase type

MIC ($\mu g/ml$)	DMPPC	ABPC	TC	EM	CFDN
≦0.2	1	3	18	30	14
0.39	0	1	0	1	19
0.78	1	5	0	0	0
1.56	3	6	0	0	1
3.13	24	13	1	0	0
6.25	4	3	7	0	0
12.5	1	2	5	0	0
25	0	0	3	0	1
50	0	1	0	0	0
100	0	1	1	1	0
>100	1	0	0	3	0

coagulase type	I	II	III	IV	V	VI	VII	VIII	UT
No. of strains	0	9	4	2	10	0	5	2	3

35 isolates from nasal mucosa (1996)

DISCUSSION

MRSA is a major organism involved in nosocomial infection. It is recognized that the increased isolation frequency of MRSA is associated with the use of antibiotics, especially the third generation cephems. We examined *S. aureus* isolated in Lao PDR in 1993, and MRSA was not found (Higa *et al.*, 1994). According to the hospital and drug stores in the city (we got the information through the questionaire), the restricted kinds and amount of antimicrobials such as penicillin (G and V), ampicillin, tetracycline, gentamicin, erythromycin, sulfamethoxazol-trimethoprim compound and nalidixic acid, were used in the country before 1993. However, since then, the types and amount of consumed antimicrobials increased with amoxicillin, chloramphenicol, cloxacillin, doxycycline, lincomycin, and ofloxacin. The drug susceptibility of *S. aureus* looks getting resistant little by little. All 54 isolates from the infection focus in 1993 were inhibited by methicillin at the concentration of 6.25 $\mu g/ml$ including 7% of the isolates inhibited at 6.25 $\mu g/ml$, and most strains were inhibited at 3.13 $\mu g/ml$. However, 37% of the 32 isolates in 1995 were inhibited at 6.25 $\mu g/ml$, and there was no strain to which the MIC of methicillin was 12.5 $\mu g/ml$ or more. In 1996, 42% of the 47 isolates were inhibited at 6.25 $\mu g/ml$ or more, including one strain inhibited at 12.5 $\mu g/ml$ of methicillin. However, the 35 isolates (in 1996) from non-infected patients and the nurses working at the wards included 4 strains (11%) inhibited at 6.25 $\mu g/ml$, one strain at 12.5 $\mu g/ml$, and one highly resistant strain (resistant at 100 $\mu g/ml$). This highly resistant strain possessing *mecA* gene is the first reported MRSA in Lao PDR.

The coagulase types of MRSA recently isolated in Japan were mostly type II, but that of the first reported MRSA from Lao PDR was type IV. It reminds us of the fact that, in the beginning of 1980s when the problem of MRSA was recognized in Japan, the coagulase type of MRSA was mainly type IV (Matsumoto *et al.*, 1984). The small percentage of coagulase type II in Lao PDR may be due to the rare occurrence of MRSA at present in this country.

This emergence of MRSA in Lao PDR is likely a result of the increased use of a various antimicrobials. In this study, only one isolate of MRSA was found in a non-infective patient, but not in the patient with Staphylococcal infection. It may be due to small number of parameter (Number of Patient examined), because the isolation frequency of drug resistant organism supposed to be higher in the patients with antibiotic treatment than those without antibiotics. Therefore, it is now important to start surveillance to control MRSA in this country.

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We thank the staff of Mahosot Hospital for collecting bacterial strains.

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KETOTIFEN, AN ANTIALLERGIC AGENT, SUPPRESSES PULMONARY EOSINOPHILIA INDUCED BY THE NEMATODE *NIPPOSTRONGYLUS BRASILIENSIS* IN RATS

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Abstract: Certain parasitic infections such as ascariasis and hookworm diseases occasionally cause a type of Löffler's syndrome. This syndrome, which consists of transitory and migratory pulmonary infiltrations with eosinophilia, is presumed to be triggered by hypersensitivity reaction. Previously, we showed that infection with the nematode *Nippostrongylus brasiliensis* induces similar lung lesions, that are markedly enhanced after challenge infection. To examine the role of mast cells in nematode-induced pneumonitis, *N. brasiliensis*-primed Brown Norway rats received continuous intravenous administration of the mast cell stabilizer ketotifen (6 mg/day/kg body weight) from one day prior to 3 days after challenge infection. Significantly larger numbers of rat mast cell protease (RMCP) II-positive mast cells were identified in the lungs of ketotifen-administered animals than in saline-perfused animals, suggesting that ketotifen treatment significantly suppressed mast cell activation. Morphological analysis of lung sections showed that ketotifen administration significantly suppressed eosinophil infiltration. Areas of lung granulomas, also triggered by nematode infection, were smaller in ketotifen-treated than in non-treated animals, although this effect was not statistically significant. The worm recoveries from the small intestine did not differ between ketotifen-treated and non-treated animals, indicating that the agent did not hinder lung migration of worms. These results suggest that mast cells play an important role at least in the development of mematode-induced lung eosinophilia.

Key words: eosinophil, mast cell, lung, rat, *Nippostrongylus*, nematode, ketotifen, Löffler's syndrome

INTRODUCTION

Infection with certain intestinal nematodes, such as *Ascaris lumbricoides, Strongyloides stercoralis* and hookworms occasionally causes pulmonary infiltration with peripheral blood eosinophilia recognized as a type of Löffler's syndrome (Gelpi and Mustafa, 1968; Phills *et al.*, 1972; Spillmann, 1975). The lung lesions appear to be associated with migrating nematode larvae, as the larvae transiently pass through the lungs and trachea before infesting the final habitat, the intestine. These patients usually have elevated IgE antibody levels and symptoms consisting of dyspnea, often of an asthmatic type, suggesting that hypersensitivity mechanisms might be involved in the development of nematode-associated lung lesions (Phills *et al.*, 1972). Like these human parasites, infection with the rodent intestinal nematode *Nippostrongylus brasiliensis* induces marked infiltration of eosinophils, an increase in the number of alveolar macrophages, development of granulomas, and an increase in the number of mast cells in the lungs (Talliaferro and Sarles, 1939; Arizono *et al.*, 1987; Ramaswamy *et al.*, 1991). Therefore, *N. brasiliensis* infection in rodents provides a suitable model in which to examine the mechanisms of nematode-induced pneumonitis.

In a previous study, we showed that genetically mast cell-deficient *Ws/Ws* rats exhibited significantly lower levels of eosinophil infiltration and granuloma formation in the lungs than in normal +/+ rats after *N. brasiliensis* infection, although elevated levels of IgE antibody did not differ between *Ws/Ws* and +/+ rats,

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suggesting that mast cells play an important role for triggering the lung lesions (Arizono *et al.*, 1996). In the present study, we further investigated whether an anti-allergic drug, ketotifen, would suppress the nematode-induced lung lesions in rats. Ketotifen {4-(1-methyl-4-piperidylidene)-4H-benzo[4,5]cyclohepta [1, 2-b] thiophen-10 (9H)-one hydrogen fumarate} stabilizes mast cells and shows H1-selective histamine antagonism (Martin and Roemer, 1977; Craps and Nay, 1984).

Materials and Methods

Animals and nematode infection

Specific pathogen-free, male Brown Norway (BN/Sea) rats were purchased from Seiwa Experimental Animals Co. (Fukuoka, Japan). The animals, 8 weeks old (body weight 180-190 g), received a subcutaneous injection of 2,000 infective-stage larvae of *N. brasiliensis* as described previously (Arizono *et al.*, 1996). A group of animals were sacrificed 28 days after infection. Other animals were divided in 2 groups, one subjected to ketotifen infusion and the other to saline infusion as a control (see below), and the latter 2 groups received a challenge infection with 2,000 infective-stage larvae on day 28 after the primary infection.

Passive cutaneous anaphylaxis (PCA) reaction

Naive rats were injected intradermally with 50 μl of serially diluted test sera obtained 4 weeks after *N. brasiliensis* infection or with uninfected rat sera. Forty-six hours later, these animals received intravenous injections of various doses of ketotifen. Two hours after ketotifen administration, each animal received intravenous injection of 1 mg of *N. brasiliensis* adult worm somatic antigen (Yamada *et al.*, 1991) and 100 mg of Evans blue dye dissolved in 1 ml of saline. The blueing of sensitized skin was measured 30 min later and spots more than 5 mm in diameter were regarded as positive.

Continuous intravenous infusion of ketotifen

Long-term intravenous infusion of ketotifen in unrestrained rats was carried out by the method described by Steiger *et al.* (1972) with some modifications. Animals which had been infected with *N. brasiliensis* 27 days previously were anesthetized with Nembutal® (intraperitoneal injection, 25 mg/kg) and small skin incisions were made in the occipital as well as in the right cervical region. The right jugular vein was isolated and ligated with a silk suture. A second suture was placed around the vein 1.0-1.5 cm proximal to the heart and was left untied until the catheter had been inserted. A sterilized silicone tube with a 0.5-mm inner diameter was inserted into the jugular vein between the sutures and advanced into the superior vena cava. The silicon tube was then secured with the silk sutures. The free side of the silicone tube was passed through the subcutaneous tissue into the occipital region where a skin incision had been made, and secured to the exit site using a specifically constructed stainless steel apparatus. The tube outside the animal was covered by a spring coil, and the free end of the tube was attached to a swivel from which a second tube was connected to an infusion pump (STC-501; Terumo, Tokyo, Japan). This method allowed the rat to move freely during infusion. Animals were infused with ketotifen (fumarate salt, Sigma Chemical Co., St. Louis, Mo., USA) dissolved in saline at a dose of 6 mg/day/kg, or saline as a control, at a rate of 0.2 ml/hr. Twenty-fours hour later, these animals were infected subcutaneously with 2,000 *N. brasiliensis* L3 larvae. Ketotifen or saline infusion was continued for a further 72 hr and then animals were sacrificed by overdose of ether.

Histology

Lungs were removed and fixed for 3 hr in Carnoy's fluid or for 48 hr in 4% formalin in 0.1 M phosphate buffer, pH 7.3. Formalin-fixed paraffin-embedded lung sections were subjected to staining with hematoxylin and eosin (H-E), or with carbol-chromotrope for eosinophil identification (Lendrum, 1944). Mast cells were stained in Carnoy's fluid-fixed paraffin-embedded sections for 30 min with 0.5% alcian blue dissolved in 0.5 N HCl.

Immunohistochemical staining of rat mast cell protease (RMCP) II

RMCP II immunostaining was carried out as described previously (Arizono *et al.*, 1994). Briefly, the lung tissues were fixed in phosphate-buffered 4% formalin (pH 7.3) for 48 hr. Paraffin sections were cut at 4 μm and endogenous peroxidase activity was blocked with 0.3% H_2O_2. Sections were incubated in 10% normal goat serum and then successively incubated with rabbit anti-RMCP II serum, and with horseradish peroxidase-conjugated goat anti-rabbit IgG serum (Zymed Lab. Inc., San Francisco, CA, USA). Finally, the DAB reaction was performed. As a negative control, tissue sections obtained from congenitally mast cell-deficient *Ws/Ws* rats were used.

Counts of alcian blue positive- or RMCP II positive-mast cells in lung sections

Fields of vision in the alcian blue-stained or RMCP II immunostained lung sections were transcribed to digital images using video microscopy (HC-2000D, FUJI FILM, Tokyo, Japan). Alcian blue positive- or RMCP II-positive mast cells were counted using Mac Scope image processing software (MITANI Co., Fukui, Japan). A total lung area of 5.99 mm^2 was processed per animal.

Counts of eosinophils in lung sections

Ten percent buffered formalin-fixed, carbol-chromotrope-stained lung sections (4 μm) were photographed and printed at a magnification of ×180. Eosinophils were counted on the photographs and numbers of eosinophils/mm^2 were determined. A total lung area of 0.86 mm^2 was analyzed per animal.

Measurement of lung granulomas

H-E-stained lung sections were photographed and printed at a magnification of ×13. The areas of granulomas were measured with an electric digitizer (Two-dimensional measuring software, Nikon cosmozone; Nikon, Tokyo, Japan). Granuloma areas were added and total granuloma area/mm^2 of lung section was determined.

Counts of worms in the small intestine

The small intestine was removed from infected rats, opened longitudinally and incubated at 37°C in normal saline for 3 hr. The numbers of emerging worms were counted under a dissecting microscope.

Table 1 Suppression of PCA reaction by ketotifen. Ketotifen was intravenously injected into rats which had received intradermal injection of serially diluted *N. brasiliensis*-infected rat sera 46 hr previously. These animals were challenged with intravenous injection of the worm somatic antigen 2 hr later, and PCA titers were determined. Injection of uninfected-rat sera showed PCA titers less than 2^4

Animal No.	Ketotifen (mg/kg)	Dilution of sera					
		2^4	2^5	2^6	2^7	2^8	2^9
1	0	+	+	+	+	+	−
2	0.05	+	+	+	−	−	−
3	0.2	+	+	−	−	−	−
4	0.5	+	+	−	−	−	−
5	1.0	+	+	+	−	−	−
6	2.5	+	+	+	−	−	−

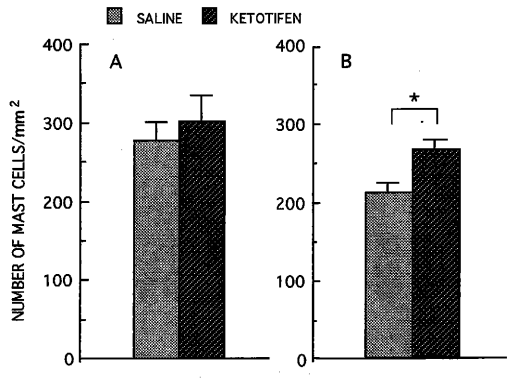

Figure 1 Numbers of alcian blue-positive (A) and RMCP II-positive (B) mast cells in the lungs of rats 3 days after challenge infection with *N. brasiliensis*. Ketotifen (6 mg/day/kg) or saline was infused from one day before to 3 days after the challenge infection. Columns and bars represent means ± SE of 4 rats. *Significantly different ($P<0.01$).

Statistical analysis

Student's t-test was used to evaluate the significance of differences.

RESULTS

The effective suppressive dose of ketotifen on immediate hypersensitivity was examined using PCA reaction. Two hours after ketotifen injection, PCA reaction was suppressed most significantly with 0.2 or 0.5 mg/kg ketotifen (Table 1). The suppressive effect of ketotifen became less distinct when ketotifen was administered 3 or 7 hr before the antigen challenge, and it was totally abolished when ketotifen was injected 24 hr before challenge, indicating that the suppressive effect of ketotifen was rather short-lived (data not shown). Thus, a continuous intravenous perfusion method was employed with ketotifen at 6 mg/day/kg, a dose corresponding to administration of 0.5 mg/kg ketotifen every 2 hr.

N. brasiliensis-primed animals were perfused continuously with 6 mg/day/kg of ketotifen from 24 hr before challenge infection to 3 days after challenge infection. The numbers of alcian blue-stained lung mast cells in ketotifen-treated animals were not significantly different from those in saline-perfused animals (Fig. 1A). In contrast, numbers of RMCP II-positive mast cells in ketotifen-treated animals were significantly larger than in control animals (Fig. 1B), suggesting that ketotifen treatment suppressed mast cell activation that allowed more mast cells to retain RMCP II in the cytoplasmic granules. In fact, high power microscopic

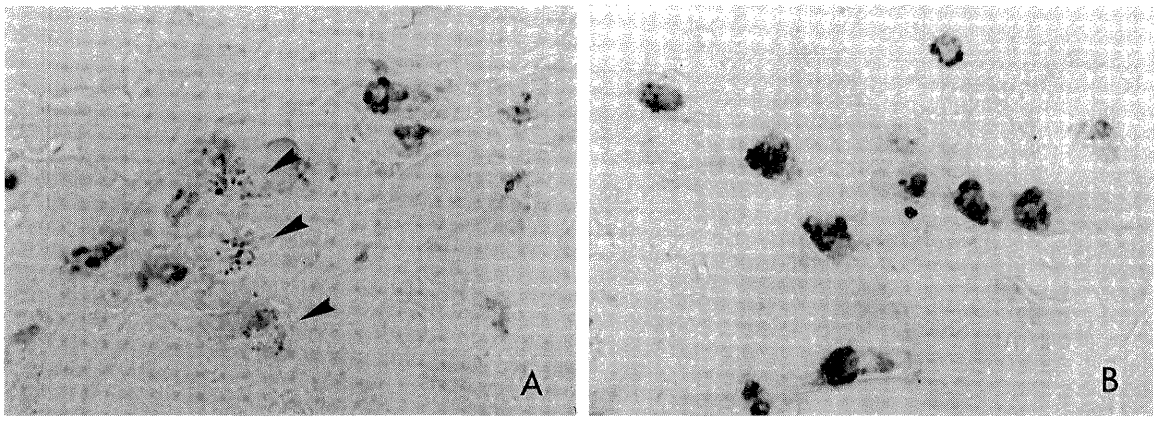

Figure 2 Immunostaining of RMCP II-positive mast cells in the lungs after challenge infection with *N. brasiliensis*. Animals received saline (A) or ketotifen (B) from one day before to 3 days after the challenge infection. In saline-perfused animals, some of the mast cells showed degranulation (arrow heads), while mast cells in ketotifen-treated animals did not. Magnification, ×600.

observations of RMCP II-immunostained lung sections frequently showed mast cell degranulation in saline-perfused animals, while not in ketotifen-treated animals (Fig. 2).

Eosinophil infiltration in the lungs was examined on carbol-chromotrope-stained lung sections. After challenge infection, numbers of eosinophils in the lungs were significantly increased in saline-perfused animals, while eosinophil numbers did not increase in ketotifen-treated animals (Fig. 3).

Lung granulomas developed after *N. brasiliensis* challenge infection (Fig. 4). The lesions consisted of histiocytic cells, fibroblasts, and lymphoid cells with occasional appearance of multinucleate giant cells. Some of the eosinophils also appeared in the granulomatous lesions. Areas of lung granulomas in ketotifen-treated animals were smaller than in saline-perfused animals, although this was not statistically significant because of the large variations in the reactions (Fig. 5).

To exclude the possibility that ketotifen might have directly affected *N. brasiliensis* and suppressed the migration of worms into the lungs, numbers of worms in the small intestine 3 days after challenge infection were counted. L4 larvae were recovered from the small intestine without significant differences in the numbers between ketotifen-treated and non-treated animals (Fig. 6). Further, *N. brasiliensis* L3 larvae and adult worms were incubated at 37°C with various doses of ketotifen dissolved in saline. Approximately 90% of adult worms showed active mobility 8 hr after incubation and 80% after 24-hr incubation regardless of the presence or adsence of ketotifen from 0 to 2 mg/m*l*. Viability of L3 larvae was also not affected in the presence of ketotifen.

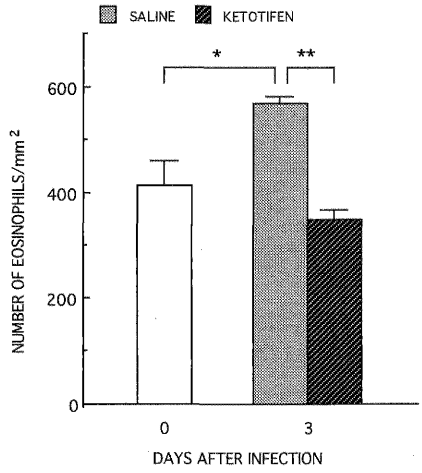

Figure 3 Numbers of eosinophils in the lungs before and 3 days after challenge infection with *N. brasiliensis*. Ketotifen (6 mg/day/kg) or saline was infused from one day before to 3 days after the challenge infection. The data before challenge infection were obtained from rats at 28 days after primary infection. Columns and bars represent means ±SE of 4 rats. Significance of differences ($*p<0.05$, $**P<0.01$).

Discussion

The principal pharmacological effect of ketotifen has been reported to be a stabilization of mast cells, prohibiting release of chemical mediators such as histamine and leukotrienes from the cells. In addition, ketotifen is an H1-selective histamine antagonist (Martin and Roemer, 1977; Craps and Nay, 1984). In the

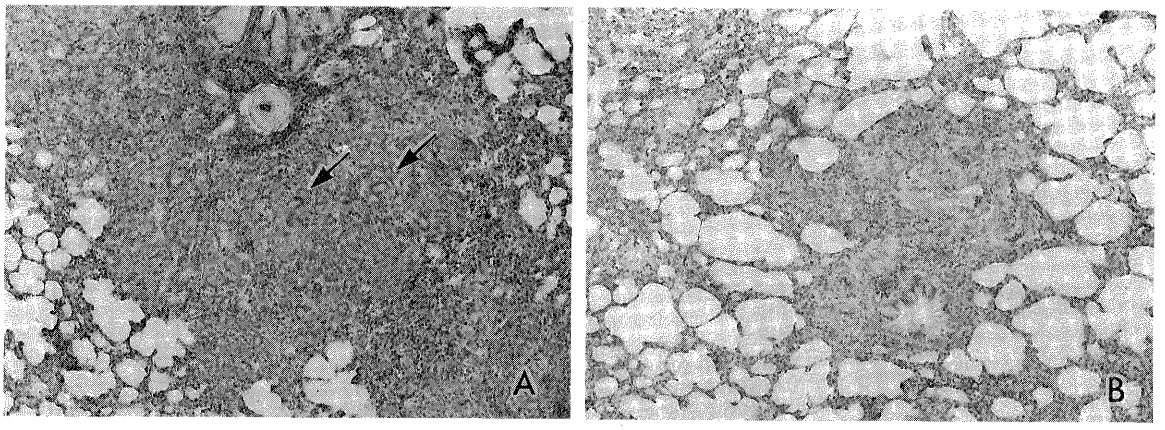

Figure 4 Granulomatous response in the lungs after challenge infection with *N. brasiliensis*. Animals received saline (A) or ketotifen (B) from one day before to 3 days after the challenge infection. Multinucleate giant cells (arrows) were observed in the granuloma. In ketotifen-treated animals, small granulomas were more frequently found than in saline-perfused animals. H-E, ×60.

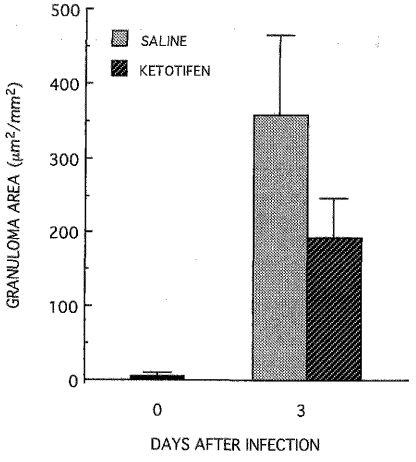

Figure 5 Granuloma areas in the lungs before and 3 days after challenge infection with *N. brasiliensis*. Ketotifen (6 mg/day/kg) or saline was infused from one day before to 3 days after the challenge infection. Data before challenge infection were obtained from rats at 28 days after primary infection. Data shown are means of total granuloma areas ±SE of 4 rats.

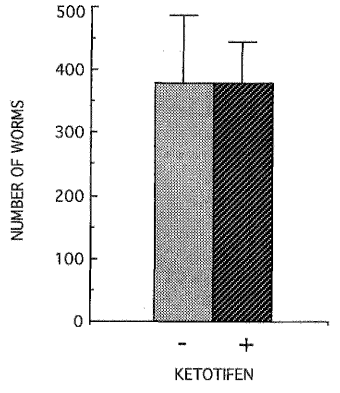

Figure 6 Worm recovery from the small intestine of rats 3 days after challenge infection with *N. brasiliensis*. Ketotifen (6 mg/day/kg) or saline was infused from one day before to 3 days after the challenge infection. The majority of recovered worms were L4 larvae. Data shown are means ±SE of 4 rats.

present study, the numbers of RMCP II-positive mast cells were significantly larger in ketotifen-treated than in non-treated animals despite the similar levels of alcian blue-positive mast cell numbers between the two groups of rats. Since RMCP II, a preformed intragranular mediator, is readily released together with histamine and other chemical mediators after mast cell activation (Woodbury *et al.*, 1984), the appearance of larger numbers of RMCP II-positive mast cells in ketotifen-treated animals appears to reflect suppression of mast cell activation by this agent. In fact, it has been reported that anaphylactic secretion of RMCP II following intravenous challenge of primed rats with worm antigen was accompanied by significant depletion of this enzyme and decreased numbers of RMCP II-positive mast cells in the tissues (King *et al.*, 1986). The present results showed that ketotifen treatment induced significant suppression of eosinophil infiltration into the lungs. Granulomatous reaction also seems to have been suppressed, although not to a statistically significant level because of the large variation in the reaction. On the other hand, the worm recoveries from the small intestine did not differ between ketotifen-treated and

nontreated animals, indicating that the agent did not hinder lung migration of worms. These results suggest that mast cells have an important role at least in the development of nematode-induced lung eosinophilia.

Mast cells are the major effector cell for type I hypersensitivity, in which IgE antibody-mediated mast cell activation induces prompt mediator release. In fact, *N. brasiliensis* infection induces marked elevation of specific IgE antibody (Yamada *et al.*, 1991). Further, *N. brasiliensis*-sensitized rat lungs showed immediate release of histamine and leukotriene C4 upon worm-antigen stimulation *in vitro* (Nishida *et al.*, manuscript in preparation), suggesting that at least in part nematode-induced pneumonitis might be an expression of type I hypersensitivity. On the other hand, it has been reported recently that Arthus reaction, the type III hypersensitivity mediated by IgG immune complexes, is also dependent on mast cells (Ramos *et al.*, 1994; Sylvestre and Ravetch, 1996). Further, mast cells also augment various non-allergic inflammatory responses (Galli, 1993). Thus, the precise pathogenetic mechanisms of the development of nematode-induced penumonitis and the role of mast cells remain to be elucidated.

Taken together, the present findings suggest that development of nematode-induced lung eosinophilia is dependent on mast cell activation, and mast cell stabilizers appear to be partially, if not completely, effective for suppression of the lung lesions.

ACKNOWLEDGMENTS

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A FIELD STUDY ON THE EFFECTS OF RESIDUAL SPRAY OF ENCAPSULATED FENITROTHION ON *ANOPHELES MINIMUS* POPULATION IN PHARE PROVINCE, NORTHERN THAILAND

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Abstract: A field study was carried out in 2 villages of Phare Province, Thailand to evaluate effect of the residual spraying of a microcapsulated formulation of 20% fenitrothion (Sumithion 20 MC®) on *Anopheles minimus* populations. In the treatment village, houses were sprayed with 1 g/m² of fenitrothion, except for 2 houses which were selected to spray with 0.5 g/m² of fenitrothion for comparative bio-assay test. The results of bio-assay test showed that mortality of *An. minimus* was 100% in 1 g/m²-30 miniutes until 4 months after the spray. The growth rate of *An. minimus* population during the first 4 months of the study period in the treatment village was lower than that in the control area. These results suggested that the residual spray of fenitrothion microcapsules at the beginning of the dry season was effective at least for 4 months after the spray and could suppress the density of *An. minimus*.
Key words: Residual spray, Fenitrothion, Microcapsule, *Anopheles minimus*, Thailand

INTRODUCTION

DDT has played an important role in the vector control until recent years in developing countries. However, the behavioral and/or physiological resistance of mosquitoes have been developed by the continuous application of DDT (WHO, 1992). It has become a serious problem in vector control in many countries, in addition to the side effects of DDT on the surrounding environment through the biological concentration (Curtis, 1994).

In Thailand DDT has been used on a large scale for the malaria vector control since 1952. The physiological and behavioral resistance to DDT have been observed in some anopheline species in Thailand (WHO, 1970, 1992). The screening of alternative insecticide to DDT has become an important subject in the vector control, though it has been believed that the major malaria vectors, *Anopheles minimus* and *An. dirus*, have been still susceptible to DDT (Ismail *et al.*, 1974, 1975; Nutsathapana *et al.*, 1986). Since 1982 fenitrothion has been introduced to some areas as another candidate of insecticide. In 1988 a field study on the response of *An. dirus* to DDT and fenitrothion was conducted and the results suggested the presence of behavioral resistance (Suwonkerd *et al.*, 1990). In this study effects of the residual spray of a microcapsulated formulation of 20% fenitrothion (Sumithion 20 MC®) on *An. minimus* were examined in northern Thailand.

MATERIALS AND METHODS

The study was carried out from October 1995 to September 1996 in 2 villages in Saeipt Canton, Song District, Phare Province, 245 km east of Chiangmai City, Thailand. One village (No. 8 Ban Tawa) where 67 houses were situated and the population=272 was selected as the treatment area. The other village (No. 5 Ban Mae Ten) was selected as the control area where 89 houses were situated and the population was 340.

Among the houses in the treatment area, 2 houses were selected to spray with 0.5 g/m² of encapsulated fenitrothion for the comparative bio-assay test and the other houses were sprayed with 1 g/m² in October, 1995.

Before the insecticide spray, mosquito collections were performed in 4 successive nights. Monthly mosquito collection (4 nights) was conducted by 3 different methods for 1 year after the spray. Using human baits,

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2 different collections, indoor and outdoor human bait collections were made from 18:00 to 24:00 at 2 fixed houses. A pair of collectors sat inside and outside of the houses and landing mosquitoes were collected by an aspirator hourly for 50 min. In the 3rd collection method (the animal bait collection), a cow was tethered inside a gauze net (4×4×2 m), which was similar to the one described by Service (1993), and one collector caught mosquitoes landing in and out of the net using an aspirator at every 15 min. During the collection period relative humidity and temperature were recorded.

Following WHO (1970) the bio-assay test was made every month for 2 different dosage (0.5 and 1 g/m² of fenitrothion) and 2 different periods of exposure (3 and 30 min) using 100-200 *An. minimus* adults reared in the laboratory. The test was duplicated in each combination.

For the bio-assay test the mortality was calculated for each replication and the average of the 2 replications was shown in the table. The average number of *An. minimus* per half night was calculated for each collection method and the growth rate of *An. minimus* population during the first 4 months of the study period was estimated by applying log regression analysis to the average number of *An. minimus*+1.

Table 1 The result of bio-assay test of *An. minimus* on the residual deposits of 20% microcapsulated fenitrothion sprayed from October 1995 to September 1996 in Phare, Thailand

Time after spray (month)	0.5 g/m²*		1 g/m²*	
	3 min**	30 min**	3 min**	30 min**
0	100	100	100	100
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	9	49	52	100
5	7	44	12	95
7	9	40	14	78
9	10	25	17	47
10	11	35	15	48
11	14	34	18	40

The total number of mosquitoes used in each test was 100-200 and mortality rate of the control mosquitoes was 0 in all the bio-assay test.

* Dosage of fenitrothion

** Exposure time

Results and Discussion

The results of bio-assay test showed that mortality of *An. minimus* was 100% until 3 months after the spray in all combinations (Table 1). Only the combination of the higher dosage with 30 min exposure showed 100% of mortality until 4 months after the spray. The difference in the effective period between 2 dosage, 0.5 g/m² and 1 g/m², was about 1 month, and the higher dosage always showed the higher mortality.

The temporal changes in the number of *An. minimus* collected by 3 different methods are depicted in Fig. 1. The density of *An. minimus* declined after the insecticide spray, except for the result of animal bait collection in the control area, and started to increase in May 1996. Although the observed temporal changes in the density may be partly ascribed to the seasonal prevalence of this species in northern Thailand (Takagi *et al.*, 1995; Suwonkerd *et al.*, 1995), the density of indoor and outdoor collection in the treatment area decreased more rapidly from November to December 1995 than the control area.

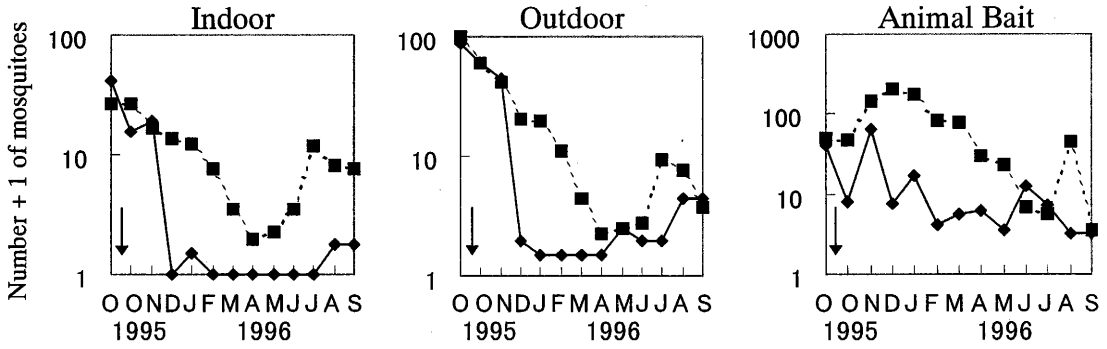

Figure 1 Temporal changes in the number of *An. minimus*/half night collected by 3 different methods from October 1995 to September 1996 in 2 villages in Phare, Thailand. The solid and dashed line shows the result in the treatment and control village, respectively. The arrow shows the day of residual spraying of 1 g/m² of microcapsulated fenitrothion in the treatment village.

Table 2 Results of log regression analysis of the temporal changes in the number of *An. minimus* collected by 3 different methods in the control and treatment area* from October 1995 to February 1996, Phare, Thailand

	Indoor		Outdoor		Animal Bait	
	Treatment	Control	Treatment	Control	Treatment	Control
Slope (b)	−0.35	−0.123	−0.468	−0.18	−0.111	0.059
S.E.	0.126	0.017	0.132	0.023	0.153	0.09
Growth rate/ month (10^b)	0.447	0.753	0.340	0.661	0.775	1.146
Correlation coefficient (r)	0.721	0.944	0.808	0.953	0.149	0.125

*Houses were sprayed with 1 g/m² of microcapsulated fenitrothion in October 1995.

Because the bio-assay test showed that the sprayed insecticide was effective at least for the first 4 months of the study period, the growth rate of the average number of *An. minimus* during the first 4 months (from October 1995 to February 1996) was estimated and compared in Table 2. Both indoor and outdoor collections, the slope of log regression line (b) in the treatment area was significantly smaller than that in the control area. The growth rate in the control area was estimated as 0.753 and 0.661/month for indoor and outdoor collections, respectively, and that in the treatment area was 0.447 and 0.340/month for indoor and outdoor collections, respectively. Though the log regression was not significant in the animal bait collection, the difference between the treatment and control area was clear; the density of *An. minimus* in the control area showed gradual increase whereas that in the treatment area showed decreasing tendency during the first 4 months of the study period (Fig. 1).

All the results suggested that the residual spray of capsulated fenitrothion at the beginning of the dry season was effective at least for 4 months after the spray and could suppress the density of *An. minimus*. However the present study started from the declining period of *An. minimus* population, thus, the effect of the insecticide sprayed might be overestimated in this study. Additional field studies in the increasing period of *An. minimus* population will be needed to evaluate the effect of the residual spray of microcapsulated fenitrothion in malaria vector control throughout the year.

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二酸化塩素溶液の *Cryptosporidium* のオーシストに対する保存効果

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緒　　論

Cryptosporidium はコクシジウム類の中で最も小さく，一般にオーシストの検出には蔗糖液浮遊法が用いられている（Current, 1990; Fujino *et al.*, 1996）。しかし，オーシストは乾燥や熱処理を受けると蔗糖液でも浮遊せず，検出困難となる。さらにオーシストの生存には酸素が要求される（Davis, 1973）ことから，その保存には酸素を消費する糞便内の細菌を殺滅することが必要である。このために通常実験室内では 2%から2.5%の重クロム酸カリ溶液中で保存されているが，クロムは産業廃棄物であることから環境汚染を防ぐために特別な廃棄処理が要求され（Current, 1990），安全で簡単に廃棄できる保存液の開発が望まれる。

そこで，某薬品会社が畜舎用消臭剤として，住宅や畜舎の消毒・消臭用に販売している水成二酸化塩素溶液（商品名「FC1736」），および重クロム酸カリ溶液の代用品として使用されている希硫酸（Frenkel and Dubey, 1975; Matsui, 1991）とについて，*Cryptosporidium muris* とニワトリ由来 *Cryptosporidium* sp. とのオーシストの保存効果を調べた。

材料および方法

供試原虫：

C. muris のオーシストは自然感染ドブネズミの糞便から分離し（Iseki, 1986），マウスで継代・増殖したものを，またニワトリ由来 *Cryptosporidium* sp. のオーシストは自然感染鶏の糞便から採集し（Itakura *et al.*, 1984），その実験感染鶏のファブリキウス嚢より分離した（松井ら，1992）後，ニワトリで継代・増殖したものをそれぞれ供試した。

供試動物：

4週齢のICR雄のマウスと1週齢の白色レグホンの雄を供試した。いずれも実験前に数回蔗糖液浮遊法による糞便検査を行い，オーシスト陰性であることを確認した後 coccidia-free の管理下で飼育した。

供試保存液：

オーシストの保存効果を調べたFC1736は，水成二酸化塩素溶液より製剤化された商品（ClO_2．Na_2CO_3）で，その1%と2%溶液を供試した。希硫酸は2%溶液を供試し，2%の重クロム酸カリ溶液を対照として用いた。

検査方法：

C. muris またはニワトリ由来のオーシストを経口投与後，新たなオーシストを排泄中のマウスまたはニワトリの新鮮な糞便を採集し，水を加えてよく攪拌した。これを50と100の二重メッシュで濾過し，濾液を2,000 rpm 7分遠沈した後上清を捨て，沈渣にほぼ同量の水を加えて攪拌した。この材料に7倍量の供試保存液を加えて混和した後，冷蔵庫内で保存した。

C. muris は1，2，3週間および1，2カ月間，ニワトリ由来のオーシストは1および3週間放置後，水で6回遠心洗浄し，*C. muris* は1群5頭のマウスに1頭当たり 2.0×10^6 個のオーシストを，またニワトリ由来のものは1群3羽のニワトリに1羽当たり 3.0×10^6 個のオーシストをそれぞれ経口投与した。保存効果は，実験動物の糞便1g中のオーシスト数（OPG値）を毎日計測し（松井ら，1992，1994），その推移により判定した。

結　　果

C. muris：

保存オーシストを投与したマウスにおけるオーシストの排泄状況を比較するために，2%重クロム酸カリ溶液で3週間保存したオーシストを各10^2個から10^6個投与したマウスにおけるOPG値の推移（松井ら，1994）をFig. 1のAに示した。1週間保存のオーシストを投与したマウスでは，いずれの群も10^6個台投与における典型的なOPG値の推移，すなわち，10日目に10^5個台を，その後下降した後14日目には再び上昇して10^5-10^6個台を示した（Fig. 1，B）。2週間保存では二酸化塩素溶液の2%群のみが若干低下して，10^5個台投与時と10^6個台投与時との中間の推移を，3週間保存ではさらに希硫酸群が低下して，10^5個台投与時におけるOPG値の推移を示した（Fig. 2）。

1カ月間保存では二酸化塩素溶液の1%が10^5個台投与時と類似した推移を示したが，重クロム酸カリ溶液群は10^6個台投与時の推移を維持した（Fig. 3，A）。2カ月間保存では，1%二酸化塩素溶液と重クロム酸カリ溶液群とが10^5個台投与時の推移を示したが，2%二酸化塩素溶液群は10^4個台投与時の推移を，希硫酸群は10^3個台投与時の推移をそ

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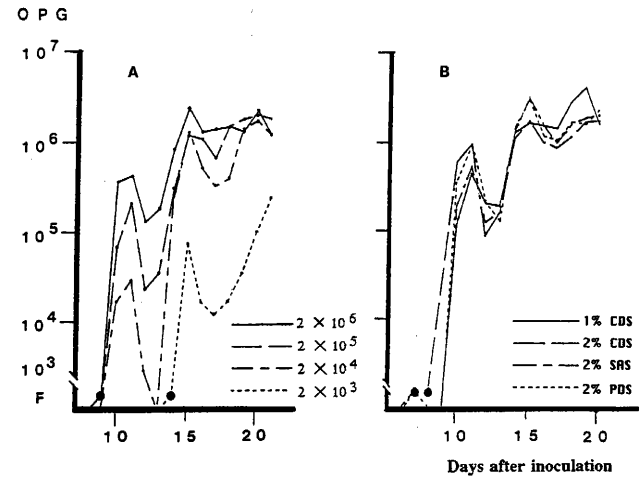

Figure 1 Comparison of mean OPG values in mice inoculated with 10^6-10^3 *C. muris* oocysts keep in PDS (A) and in mice given 2.0×10^6 *C. muris* oocysts stored in 4 storage media for 1 week (B).
F ●: Oocyst positive by sugar flotation method.
CDS: Chlorine dioxide solution. SAS: Sulfuric acid solution. PDS: Potassium dichromate solution.

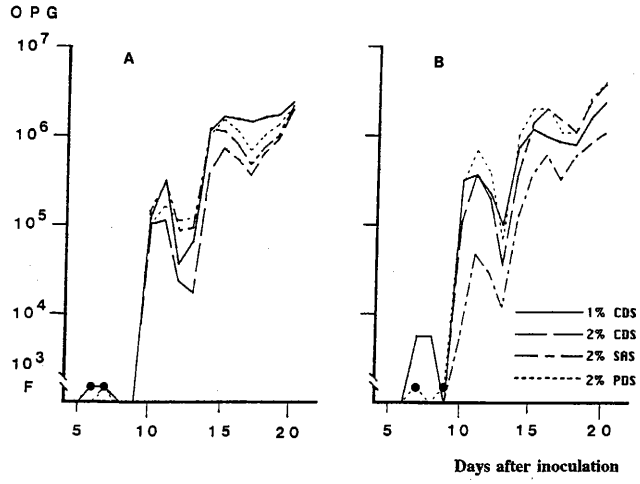

Figure 2 Mean OPG values in mice given 2.0×10^6 *C. muris* oocysts stored in 4 storage media for 2 weeks (A) and 3 weeks (B).
F ●: Oocyst positive by sugar flotation method.
CDS: Chlorine dioxide solution. SAS: Sulfuric acid solution. PDS: Potassium dichromate solution.

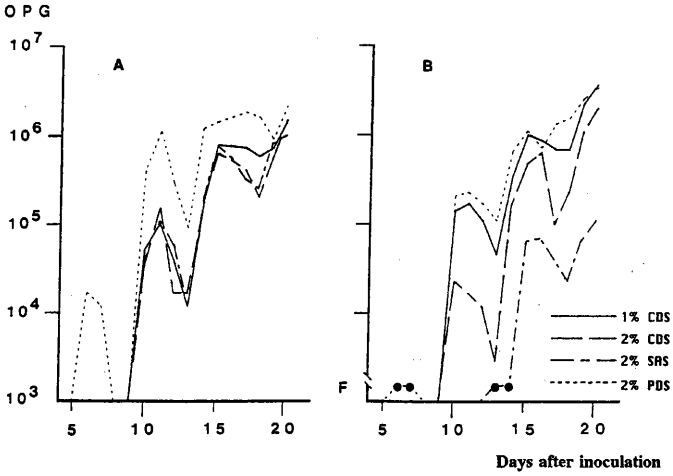

Figure 3 Mean OPG values in mice given 2.0×10^6 *C. muris* oocysts stored in 4 storage media for 1 month (A) and 2 months (B).
F ●: Oocyst positive by sugar flotation method.
CDS: Chlorine dioxide solution. SAS: Sulfuric acid solution. PDS: Potassium dichromate solution.

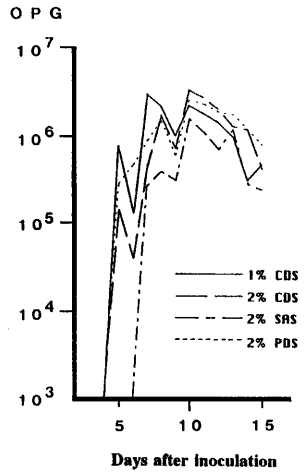

Figure 4 Mean OPG values in chickens given 3.0×10^6 chicken *Cryptosporidium* sp. oocysts stored in 4 storage media for 3 weeks.
CDS: Chlorine dioxide solution. SAS: Sulfuric acid solution. PDS: Potassium dichromate solution.

れぞれ示した（Fig. 3, B）。

ニワトリ由来 Cryptosporidium sp.：

1週間保存ではいずれも10^6個台投与時の推移を示した。3週間保存では，希硫酸群のみが若干低い OPG 値の推移を示したが，二酸化塩素溶液群は双方とも重クロム酸カリ溶液群と同様の推移を示した（Fig. 4）。

考　察

一般にコクシジウム類では宿主におけるオーシストの排泄数は接種オーシスト数に依存しており（Long, 1973），従来からその推移は宿主における感染状況の把握や治療薬の効果判定などに応用されている（Dubey and Frenkel, 1974; 飯島ら，1989; Tsunoda *et al.*, 1968）。*Cryptosporidium* についても *C. muris* は感染14日目まで，ニワトリ由来の原

虫は10日までに，接種オーシスト数と排泄オーシスト数との間に相関関係がみられている（松井ら，1992，1994）ことから，他のコクシジウム類と同様にオーシストの保存液の効果判定にも応用できる。

C. muris に対する保存液の効果を調べた結果，2％重クロム酸カリ溶液では1カ月間保存まで，1％水成二酸化塩素溶液では3週間保存まで，2％希硫酸では2週間まで，2％水成二酸化塩素溶液では1週間まで，その投与動物におけるOPG値の推移に変化が認められなかった。2カ月保存では1％水成二酸化塩素溶液と重クロム酸カリ溶液群とが10^5個台投与時の推移を示し，2％水成二酸化塩素溶液群は10^4個台投与時の推移を，希硫酸群は10^3個台投与時の推移を示したことから，それぞれ約90％，99％，99.9％のオーシストが死滅していたことが示唆された。ニワトリ由来の *Cryptosporidium* sp.においては1週間および3週間保存について調べたが，希硫酸で3週間保存した群を除き，他はいずれも10^6個台投与時におけるOPG値の推移を示し，*C. muris* における成績と類似していた。一方，全実験群でオーシスト保存前と後に蔗糖液添加法（松井ら，1992，1994）を用いてその数を測定したが，いずれにも大きな差が認められず，オーシストの生死については判定できないものの，蔗糖液浮遊法によるその検出は可能であった。これらのことから，水成二酸化塩素（FC1736）の1％溶液における保存は *Cryptosporidium* のオーシストの検出に少なくとも2カ月間有効で，実験での使用には3週間まで可能であることが明らかになった。本溶剤は入手が簡単で，安全なために容易に廃棄できることを考慮すると，患者の検査用糞便の輸送などに際しての使用も好適であると考えた。

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EFFECT OF STORAGE WITH CHLORINE DIOXIDE SOLUTION ON CRYPTOSPORIDIAN OOCYST

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Effects of storage in chlorine dioxide solution (ClO_2. Na_2CO_3, 1% and 2% w/v in water) and 2% sulfuric acid solution on oocysts of *Cryptosporidium muris* and chicken *Cryptosporidium* sp. were examined. Fresh oocysts were collected from the feces of infected mice or chickens within 1 hr of oocyst shedding. They were poured into 7-fold volume of chlorine dioxide solution, sulfuric acid solution or 2% potassium dichromate solution and stored at 4°C. After 1, 2, 3 weeks and 1 and 2 months, the stored oocysts were washed 6 times with water by centrifugation and 10^6 oocysts from each storage were inoculated to a mouse or a chicken. Inoculated animals were examined daily for oocyst discharge. When stored in 1% chlorine dioxide solution, *C. muris* and chicken *Cryptosporidium* oocysts remained viable and retained infectivity for at least 3 weeks. Viable oocysts began to decrease after 1 month in 1% chlorine dioxide solution storage, though similar number of oocysts were detected by the sugar flotation method. These results clarified that 1% chlorine dioxide solution was usefull as a storage medium to keep oocysts viable for at least 3 weeks and detectable for 2 months.

Key words: cryptosporidian oocyst, chlorine dioxide, storage medium

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