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「抗毒素製剤の効率的製造方法の開発に関する研究」

分担研究報告書
レーザー粒径測定型血小板凝集計を用いたフロキュラシオン測定系の開発

分担研究者　岩城正昭
国立感染症研究所細菌第二部主任研究官

研究要旨：本年度は、抗毒素製剤を生物製剤の品質管理のツールとして用いる、フロキュラシオンによるトキソイド抗原量測定法の海外・国内での現状と改良へのニーズに対応して研究を行なった。従来のフロキュラシオン法は抗原抗体複合体の形成を肉眼で観察することを指標にしたアッセイ系であるため、観察者の主観を完全に排除することは困難であったが、本研究において複合体の形成をレーザー粒径測定型デバイスで検出することで、(1)機器測定による高い客観性、(2)測定時間の短縮、(3)測定結果の数値化・統計処理による高い定量性、を達成することができた。

A.　研究目的

抗毒素製剤は、毒素に起因する疾患などの治療だけでなく、生物製剤の品質管理にも用いられる。抗毒素を用いた品質管理法の代表的なものは、抗原抗体反応によりトキソイドなどの抗原量を定量するフロキュラシオン法である。

フロキュラシオン法により測定されたトキソイド抗原量はLf (Limit of Flocculation)という単位で表わされ、WHOのMinimum Rrequirementsおよび国内生物学的製剤基準ではこの単位が公式に用いられる[1, 2]。

Lfの測定法であるフロキュラシオン(綿状反応)法は、最適比で混合された抗原と抗体が最も急速に抗原抗体複合体を形成することを利用している。現行法は、1923年のRamonらによる最初の記述同様、肉眼で観察しうる抗原抗体複合体粒子の形成を指標にしているため、測定結果が観察者の主観、視力に影響されやすい。

本研究では、より客観的で定量性の高いフロキュラシオンアッセイ系の構築をめざし、複合体形成の検出にレーザー粒径測定型血小板凝集計を用いる測定系の開発を試みた。

B.　研究方法

フロキュラシオンにより生じた抗原抗体複合体の形成を測定するデバイスとし

て、興和(株)製PA-20型レーザー粒径測定型血小板凝集計を用いた。トキソイドは国内二社から供与されたジフテリアトキソイドを、抗毒素はフロキュラシオン用国内参照ジフテリア抗毒素を用いた。一定濃度のトキソイド溶液に様々な濃度の抗毒素溶液を加えて(Ramon法)、凝集計内でインキュベーションしながら、直径9μm以上の抗原抗体複合体粒子の数を毎秒測定しコンピューターに記録した。

C. 研究結果

1．抗原抗体複合体の検出： 約25 Lf/mlのトキソイドと、25 u/mlの抗毒素濃度を混合し、直径9μmから34μmの粒子を検出する設定で測定を行なったところ、37℃でインキュベーション開始後約4分で複合体粒子が検出され始め、以後約2分間、粒子数は直線的に増加し、抗原抗体複合体の検出が可能であることが示された。繰り返し測定の結果はよく一致していた（図1）。

2．定量的測定： 25 u/ml ±10%、±25%の抗毒素溶液と約25 Lf/mlのトキソイドを混合してそれぞれ測定を行なったところ、抗毒素濃度が25 u/mlから離れるに従って複合体の形成が遅れた(図2)。このことから、本検出系を用いてトキソイド抗原量の定量的測定が可能であることが示唆された。

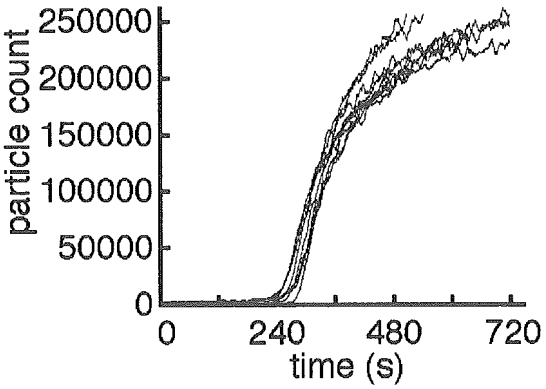

図1．粒子数の増大

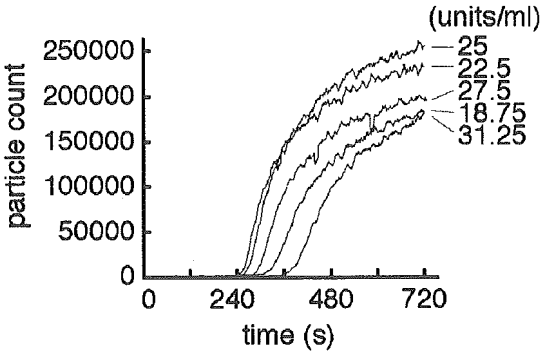

図2．定量的測定

3．統計的解析： 粒子数が直線的に増加する範囲内に閾値（50000粒子）を設定し、図2に示したそれぞれの粒子数増加曲線において、この5段階の閾値に達するまでの時間を測定し、抗毒素濃度と時間をそれぞれ横軸と縦軸に対数目盛でプロットしたところ、回帰曲線に放物線の当てはめが可能であった（図3）。

またこの放物線回帰への当てはめの妥当性を分散分析により確認した。

この回帰曲線の極小値を与える濃度の抗毒素がトキソイドと最適比を構成することを用いてトキソイド抗原量の定量を行なったところ、現行法とよく一致する結果が得られた（表1）。

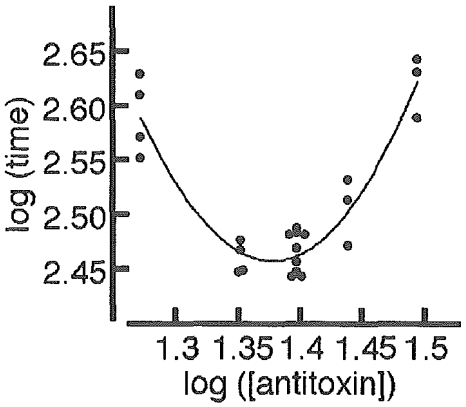

図 3. 放物線回帰

表 1. 従来法（裸眼）とレーザー法の比較

方法	トキソイド抗原量 (Lf)
従来法 (6 回測定の平均)	196.9
レーザー粒系測定法 (図 3 の回帰より計算)	190.8

D. 考察

本年度の研究で開発した、レーザー粒計測定型血小板凝集計を用いたフロキュラシオン測定系は、国内、海外における抗毒素を用いたトキソイドワクチンの品質管理法の改良に大きく貢献する可能性があることが示された。従来の方法は 1922 年に最初に記述されてから基本的に変わらない、肉眼観察を指標とした方法で、客観性の低さが問題であった。とりわけ、複合体の形成が観察されるまでの時間（Kf）が観察者によって大きく異なっていたが、レーザー粒径測定型デバイスによる検出径を用いることで、この点は大きく改善された。測定結果の定量化と統計的処理も容易であり、測定結果の詳細な解析が可能になった。今後の応用が期待される。

E. 結論

２年度目から参加した本分担研究は、抗毒素の品質管理に関する調査・研究に始まり、２年度目の海外調査により、抗毒素がツールとして品質管理に広く応用されていること、にもかかわらずその利用方法が 80 年前と基本的に変わらぬことが判明し、国内・海外で長年の課題であった「フロキュラシオン測定系」の改良に成功することができた。この成果を国際的に発信することで国際的な動きに発展することが望まれる。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

特許取得

なし

実用新案登録

なし

その他

なし

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Ⅲ．研究成果の刊行に関する一覧表

Ⅲ. 研究成果の刊行に関する一覧表

雑誌

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Fukuda T., Iwaki M., Hong,SH, Oh,HJ., Zhu,W., Morokuma,K., Ohkuma,K., Lei, D., Arakawa,Y and Takahashi,M.	Standardization of Regional Reference for Mamushi(*Gloydius blomhoffii*) Antivenom in Japan, Korea, and China	Jpn.J.Infect Dis.	59	20-24	2006
Suzuki S.,Yamazaki T.,Narita M.,Okazaki N.,Suzuku I.,Andoh T.,Matsuoka M.,Kennri T.,Arakawa Y. and Sasaki T.	Clinical Evaluation of Macrolide-Resistant *Mycoplasma pneumoniae*	Antimicrob. Agents Chemother.	50	709-712	2006
Seki N.,Sasaki T.,Sawabe K..,Sasaki T.,Matsuoka M.,Arakawa Y.,Marui E..and Kobayashi M.	Epidemiological Stadies on *Bartonella quintana* Infections among Homeless People in Tokyo,Japan	Jpn.J.Infect Dis.	59	31-35	2006

Ⅳ. 研究成果の刊行物・別刷

Jpn. J. Infect. Dis., 59, 20-24, 2006

Original Article

Standardization of Regional Reference for Mamushi (*Gloydius blomhoffii*) Antivenom in Japan, Korea, and China

Tadashi Fukuda*, Masaaki Iwaki, Seung Hwa Hong[1], Ho Jung Oh[1], Zhu Wei[2], Kazunori Morokuma[3], Kunio Ohkuma[3], Lei Dianliang[4], Yoshichika Arakawa and Motohide Takahashi

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011; [3]First, Production Department, Chemo-Sero-Therapeutic Research Institute, Kumamoto 860-8568, Japan; [1]Korea Food and Drug Administration, Soul 122-704, Korea; [2]Shanghai Institute of Biological Products, Shanghai 200052; and [4]Department of Serum, National Institute for the Control of Pharmaceutical and Biological Products, Beijing 10050, People's Republic of China

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SUMMARY: The mamushi (*Gloydius blomhoffii*) snakes that inhabit Japan, Korea, and China produce venoms with similar serological characters to each other. Individual domestic standard mamushi antivenoms have been used for national quality control (potency testing) of mamushi antivenom products in these countries, because of the lack of an international standard material authorized by the World Health Organization. This precludes comparison of the results of product potency testing among countries. We established a regional reference antivenom for these three Asian countries. This collaborative study indicated that the regional reference mamushi antivenom has an anti-lethal titer of 33,000 U/vial and anti-hemorrhagic titer of 36,000 U/vial. This reference can be used routinely for quality control, including national control of mamushi antivenom products.

INTRODUCTION

Snakebites are a threat to human life in areas inhabited by poisonous snakes. Various antivenom products have been used for the treatment of snakebites (1). Venomous snakes belonging to "mamushi" species inhabit countries in the Far East Asia, including China, Korea, and Japan (2). The mamushi species, *Gloydius blomhoffii*, is widely distributed throughout Japan, and its variants also inhabit China and Korea (*Gloydius blomhoffii brevicaudus*). These snakes were newly proposed to be regrouped into *Gloydius* spp. from *Agkistrodon* spp. in 1997 (2), and their venoms were shown to have very similar immunological characteristics. Although fatality rates of mamushi bites are generally low (3), severe cases can be lethal with cardiac, pulmonary, and/or renal dysfunction (3,4). These symptoms are caused by the snake's venom, which has lethal and hemorrhagic activities (5). Passive immunization against the venom is crucial in the clinical treatment of bites. Antivenom products can neutralize both lethal and hemorrhagic activities of the venom. In Japan and China, these products are manufactured domestically, while in Korea they are imported from Japan and China after confirmation of their potency against venom prepared from mamushi captured locally. The quality of the products has been controlled according to the minimum requirements prescribed in each of these three countries (6-8). However, the lack of international standards from the World Health Organization (WHO) precludes comparison of potency among these three Asian countries. Thus, a common reference antivenom was prepared in these countries and confirmed to be suitable as a regional reference antivenom.

In the present study, the potency of a candidate regional reference mamushi antivenom produced by Shanghai Institute of Biological Products (SIBP) (Shanghai, China) was calibrated against Japanese national standard mamushi antivenom using the quality control test methods described in the Japanese minimum requirements at the National Institute of Infectious Diseases (NIID) (Tokyo, Japan) and Chemo-Sero-Therapeutic Research Institute (Kaketsuken) (Kumamoto, Japan) and in the Korea minimum requirements at the Korea Food and Drug Administration (KFDA) (Seoul, Korea). The reference antivenom will be used in routine quality control tests in these countries.

MATERIALS AND METHODS

Production of a candidate regional reference mamushi antivenom: The candidate regional reference mamushi antivenom (Lot 011201, 3,000 vials) was produced at SIBP according to the procedure for Chinese commercial antivenom products. The candidate was made from pooled horse serum containing a sufficient antibody titer against mamushi venom. The venom and toxoid (venom detoxified with formaldehyde) prepared from Chinese mamushi were used as antigen to immunize the horse. To ensure stability of quality under storage for long periods, the candidate was freeze-dried similarly to Japanese national standard mamushi antivenom.

Animals: Mice (more than 3 mice/group; body weight, approximately 16 g) were used for determination of anti-lethal titer. The mouse strains were Slc:ddY at NIID and Kaketsuken, ICR at KFDA, and Kunmin at SIBP. For determination of anti-hemorrhagic titer, two rabbits (Japanese white strain) weighing approximately 2.5 kg were used in three countries.

Determination of anti-lethal titer: The antibody titer of the candidate regional reference material against the lethal activity of mamushi venom was determined at SIBP, NIID,

*Corresponding author: Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Tel: +81-42-561-0771 ext. 545, Fax: +81-42-561-7173, E-mail: yfukuda@nih.go.jp

Table 1. Composition of assay mixtures for mamushi antivenom titer determination

	preparation	(1)	(2)	(3)	(4)	(5)
antivenom	200 U/ml (anti-lethal test) or 20 U/ml (anti-hemorrhagic test)	0.720	0.600	0.500	0.417	0.347
PBS-G[1)]		0.280	0.400	0.500	0.583	0.653
venom	10 test dose/ml	1.000	1.000	1.000	1.000	1.000

[1)]: 0.017 mol/l phosphate buffered sodium chloride solution containing 0.2 w/v% gelatin (pH 7.0). (ml)

Kaketsuken, and KFDA, using Japanese national standard mamushi antivenom (Lot C-48, anti-lethal titer: 2,100 U/vial, NIID) with the test methods prescribed in China (8), Japan (NIID and Kaketsuken) (6,9,10), and Korea (7).

The Japanese standard antivenom was dissolved in 0.017 M phosphate buffered sodium chloride solution containing 0.2 w/v% gelatin (pH 7.0) (PBS-G) to make a solution of 200 U/ml and serially diluted with PBS-G such that 1 ml of each dilution contained 160, 125, 100, 80, or 64 U of antivenom (Table 1). The candidate (Lot 011201) was dissolved and serially diluted in a manner similar to the Japanese standard antivenom. Japanese mamushi test venom (Lot 3-2, lethal titer: 530 test dose/ampoule, or Lot 4, lethal titer: 450 test dose/vial) was reconstituted and diluted in PBS-G to a concentration of 10 test doses/ml. Aliquots of 1 ml of appropriately diluted Japanese standard antivenom or the candidate (Table 1) were mixed with 1 ml of venom and kept at room temperature for 1 h. Mice were injected intravenously with 0.2 ml of each mixture at NIID, KFDA, and Kaketsuken, and intraperitoneally at SIBP. The number of deaths was recorded for 2 days at NIID, KFDA, and Kaketsuken, or for 3 days at SIBP. The 50% effective doses (ED_{50}) of the standard antivenom and the candidate were calculated by the probit method from the number of dead mice associated with each dilution. The potency of the candidate was determined relative to that of the standard antivenom.

Determination of anti-hemorrhagic titer: The potency of the candidate regional reference mamushi antivenom was examined against the Japanese national standard mamushi antivenom (Lot C-48, anti-hemorrhagic titer: 3,300 U/vial, NIID) as standard at SIBP, NIID, and Kaketsuken using the Japanese method (6,9,11) and at KFDA using the Korean method (7).

The methods described in Japanese (6) and Korean (7) minimum requirements are essentially identical: the standard antivenom was dissolved in PBS-G to a concentration of 20 U/ml and serially diluted with PBS-G such that 1 ml of the dilution contained a total of 16, 12.5, 10, 8, or 6.4 U (Table 1). The candidate (Lot 011201) was dissolved and serially diluted in the same way as the standard antivenom. Japanese mamushi test venom (Lot 3-2, hemorrhagic titer: 1,200 test dose/ampoule, or Lot 4, hemorrhagic titer: 1,200 test dose/vial) was reconstituted and diluted into PBS-G to a final concentration of 10 test doses/ml. Aliquots of 1 ml of appropriately diluted standard antivenom or candidate were mixed with 1 ml of venom and kept at room temperature for 1 h (Table 1). Thus, aliquots of 0.2 ml of these mixtures were injected intradermally into the shaved backs of two rabbits at two sites for each rabbit per mixture. Twenty-four hours after injection, the rabbits were killed by pentobarbital anesthesia and the skin was stripped off. The cross-diameters of the hemorrhagic spots were measured from the inner side of the skin.

ED_{50} was expressed in terms of number of hemorrhagic spots measuring 10 mm in average cross-diameter. The potencies were determined relative to the standard antivenom by the parallel line method (12,13).

Estimation of stability: The stability test was performed at KFDA (7). The stability of the candidate was determined by accelerated thermal degradation test. In standard routine quality control testing, the accelerated thermal degradation test is performed by keeping the antitoxin vials at 20°C, 37°C, and 45°C for 3, 6, and 9 months in triplicate. In the present study, for simplicity, only one vial was subjected to the assay at each temperature and time. The stability of the candidate was determined by comparing the antivenom potencies of vials stored at 20°C, 37°C, and 45°C against that of vials kept at 4°C.

Estimation of safety: At SIBP, the candidate was subjected to pH testing, sterility testing, and pyrogen testing for suitability as a Chinese commercial mamushi antivenom. In the pH test, the hydrogen ion concentration of the candidate was measured with a pH meter using a glass electrode. In the sterility tests, the candidate was determined for freedom from microorganisms. In the pyrogen test, the pyrogenic activity of the candidate was determined based on febrile response of the rabbit to intravenous injection of the candidate. These methods were conducted according to the procedures prescribed in the Chinese minimum requirements (8).

RESULTS

Determination of anti-lethal titer: At SIBP, the anti-lethal titer was examined three times using 10 vials of the candidate to confirm the homogeneity of the candidate preparation. The titer of the candidate was determined to be 29,450 ± 1,650 U/vial (Table 2). No significant difference in anti-lethal potency was observed among the three examinations ($P = 0.05$). Then, the titers were determined in collaboration of NIID, Kaketsuken, and KFDA. As shown in Table 3, the titers were 31,437 (95% confidence interval: 29,111 - 33,949 U/vial at NIID, 31,572 (27,066 - 36,827) U/vial at Kaketsuken, and 36,391 (32,832 - 40,335) U/vial at KFDA. The general common potency of the anti-lethal titer determined from the results of the nine tests performed at these three facilities was 32,909 (31,080 - 34,846) U/vial, which was rounded off to 33,000 U/vial.

Determination of anti-hemorrhagic titer: At SIBP, the anti-hemorrhagic titer of the candidate was determined three times using 10 vials of the candidate to confirm the homogeneity of the candidate preparation. Potency testing indicated that the anti-hemorrhagic titer was 31,000 ± 2,550 U/vial (Table 2). No significant difference in anti-hemorrhagic potency was observed among three tests ($P = 0.05$). Then, the anti-hemorrhagic titer was measured in collaboration of three facilities. The results were 34,454 (95% confidence interval: 33,112 - 35,850) U/vial at NIID (triplicate assays), 37,543 U/vial at Kaketsuken (single assay), and 36,063 (34,411-37,793) U/vial at KFDA and NIID (triplicate assays) (Table 4). From the results of seven tests performed at these

Table 2. Homogeneity for the candidate mamushi antivenom by potency tests at SIBP

vial No.	anti-lethal titer			anti-hemorrhagic titer		
	1	2	3	1	2	3
1	30,000	28,680	28,680	30,000	30,000	30,000
2	28,680	30,070	27,360	30,000	30,000	30,000
3	31,440	28,680	24,960	30,000	30,000	30,000
4	28,680	27,360	31,440	30,000	30,000	30,000
5	31,440	30,070	31,440	30,000	30,000	30,000
6	30,000	27,360	31,440	30,000	30,000	30,000
7	28,680	30,070	31,440	30,000	37,500	30,000
8	28,680	30,070	28,620	30,000	37,500	30,000
9	28,680	30,070	28,620	30,000	30,000	37,500
10	31,440	27,360	31,440	30,000	30,000	37,500
geometric mean	29,770 ± 1,200	28,980 ± 1,180	29,560 ± 2,140	30,000	31,500 ± 3,000	31,500 ± 3,000
general geometric mean		29,450 ± 1,650			31,000 ± 2,550	

Potency tests were performed using 10 vials in triplicate. (U/vial)

Table 3. Collaborative study for anti-lethal titer determination

facility	test	potency	95% confidence interval
Japan NIID[1]	1	30,594	
	2	31,999	
	3	31,328	
	common potency	31,437	29,111 - 33,949
Japan Kaketsuken[2]	1	36,310	
	2	29,036	
	3	27,342	
	common potency	31,572	27,066 - 36,827
Korea KFDA[3]	1	31,256	
	2	46,114	
	3	37,638	
	common potency	36,391	32,832 - 40,335
general common potency		32,909	31,080 - 34,846

(U/vial)

[1]: National Institute of Infectious Diseases.
[2]: The Chemo-Sero-Therapeutic Research Institute.
[3]: Korea Food and Drug Administration.

Table 4. Collaborative study for anti-hemorrhagic titer determination

facility	test	potency	95% confidence interval
Japan NIID	1	37,847	
	2	33,033	
	3	29,561	
	common potency	34,454	33,112 - 35,850
Japan Kaketsuken		37,543	
Korea KFDA	1	42,607	
	2	35,402	
	3	42,607	
	common potency	36,063	34,411 - 37,793
general common potency		36,226	35,440 - 37,030

Abbreviations are in Table 3. (U/vial)

three facilities, the general common potency anti-hemorrhagic titer was 36,226 (35,440 - 37,030) U/vial, which was rounded off to 36,000 U/vial.

Estimation of stability: The results of the accelerated thermal degradation test performed at KFDA are shown in Table 5. The titers of the candidate stored at 20°C, 37°C, and 45°C for 9 months were 92.9, 83.1, and 83.1% of that stored at 4°C, respectively. There were no statistically significant differences between potencies and regression coefficients of the candidates stored at these temperatures for these periods ($P = 0.05$). These results indicated that there was no significant loss of anti-lethal activity of the candidate with increasing storage temperature or storage time.

Estimation of safety: The pH 6.9 determined by pH test was within the range (6.8 - 7.4) described in the Chinese minimum requirements (8). In the sterility test, there was no evidence of microbial growth. Thus, the antivenom candidate met the requirements of the test for sterility (8). The results of the pyrogen test fulfilled the requirements (8) regarding pyrogenicity.

DISCUSSION

For quality control of biological products, such as antivenoms against the bites of poisonous snakes with restricted geographical distributions or those with wider distributions but with significant geographical variation in venom activity, the WHO recommends the establishment of standard materials by individual country or by region (eastern Asia, etc.), because of the difficulty in coverage of such a wide variety of snake venoms by WHO (14). Standard mamushi antivenom is one such case. Thus, we established a regional reference mamushi antivenom for common use in China, Korea, and Japan. The antivenom raised against the venom of the snakes from China is capable of completely neutralizing those of the same species from Korea and Japan (15). Thus, we chose Chinese mamushi venom as an immunogen and the regional reference antivenom was produced in SIBP in China. From the viewpoint of improving the accuracy of the quality control test, a standard material was required to have characteristics similar to a commercial antivenom. This candidate was prepared at SIBP using the same manufacturing procedure as used for the commercial antivenom. The safety tests performed at SIBP indicated that the candidate had characteristics similar to the commercial products. The results of the stability tests performed at KFDA indicated that the candidate was sufficiently stable on long-term storage. The candidate showed an anti-lethal activity titer of 33,000 U/vial and an anti-hemorrhagic activity titer of 36,000 U/vial. The three participating countries evenly shared 3,000 vials of products (1,000 vials/country) and they will be used in

Table 5. Stability for the candidate mamushi antivenom by accelerated thermal degradation test at KFDA

	stored at (°C)	stored for 3	6	9	common (months)
Potency[1] 95% confidnce interval	20	0.825 (0.635-1.011)	0.830 (0.974-0.974)	0.929 (0.799-1.078)	0.870 (0.792-0.954)
	37	0.710 (0.544-0.866)	0.860 (0.743-0.997)	0.831 (0.715-0.964)	0.818 (0.747-0.964)
	45	0.872 (0.663-1.098)	0.837 (0.717-0.971)	0.831 (0.715-0.964)	0.840 (0.766-0.921)
	common	0.796 (0.703-0.902)	0.843 (0.775-0.916)	0.862 (0.794-0.936)	0.842 (0.799-0.888)
Regression coefficient[2]	20	10.716	14.246	12.834	12.281
	37	11.003	17.363	12.722	12.090
	45	8.959	16.411	12.722	11.681
	common	10.213	15.954	12.760	12.386

[1]: Anti-lethal titers relative to the candidate stored at 4°C for the same period.
[2]: Regression coefficient of potency.

routine quality control tests in the region.

The anti-lethal titers of the commercial products are determined in all of Japan, Korea, and China as lot-release tests. The major difference in the methods used for anti-lethal titer determination among these three countries is the route of administration of neutralized venom into mice: intravenous administration is employed in Korea (7) and Japan (6), while intraperitoneal administration is performed routinely in China (8). The route of administration was not the subject of calibration in the present collaborative study, with each country following its own method. This study design was designed based on practical considerations concerning the routine use of the product, and we found no significant differences in the results related to route of administration. However, the interchangeability of route of administration should be confirmed in future studies. Titer determination against hemorrhagic activities of venom is performed routinely for quality control testing in Japan and Korea. However, anti-hemorrhagic titer determination is not recommended by the WHO from the viewpoint of animal welfare (14). Thus, whether anti-hemorrhagic titer determination is necessary should be discussed further.

This study is the first to establish a regional reference antivenom as recommended by the WHO (14). Venomous snakes, spiders, and cnidarians inhabit restricted regions or areas, and various antivenom products have been used on a regional basis for treatment of their bites and stings. Regional reference antivenoms are required for quality control of such antivenom products. Thus, this international collaborative study will provide important information and experience for the establishment of regional reference standards.

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Clinical Evaluation of Macrolide-Resistant *Mycoplasma pneumoniae*

Satowa Suzuki,[1] Tsutomu Yamazaki,[2] Mitsuo Narita,[3] Norio Okazaki,[4] Isao Suzuki,[5] Tomoaki Andoh,[5] Mayumi Matsuoka,[1] Tsuyoshi Kenri,[1] Yoshichika Arakawa,[1] and Tsuguo Sasaki[1]*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[1] Department of Pediatrics, Saitama Medical School, Saitama,[2] Sapporo Tetsudo Hospital, Hokkaido,[3] Kanagawa Prefectural Institute of Public Health, Kanagawa,[4] and Department of Pediatrics, Chigasaki Municipal Hospital, Kanagawa,[5] Japan

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Macrolide-resistant *Mycoplasma pneumoniae* (MR *M. pneumoniae*) has been isolated from clinical specimens in Japan since 2000. A comparative study was carried out to determine whether or not macrolides are effective in treating patients infected with MR *M. pneumoniae*. The clinical courses of 11 patients with MR *M. pneumoniae* infection (MR patients) treated with macrolides were compared with those of 26 patients with macrolide-susceptible *M. pneumoniae* infection (MS patients). The total febrile days and the number of febrile days during macrolide administration were longer in the MR patients than in the MS patients (median of 8 days versus median of 5 days [$P = 0.019$] and 3 days versus 1 day [$P = 0.002$], respectively). In addition, the MR patients were more likely than the MS patients to have had a change of the initially prescribed macrolide to another antimicrobial agent (63.6% versus 3.8%; odds ratio, 43.8; $P < 0.001$), which might reflect the pediatrician's judgment that the initially prescribed macrolide was not sufficiently effective in these patients. Despite the fact that the febrile period was prolonged in MR patients given macrolides, the fever resolved even when the initial prescription was not changed. These results show that macrolides are certainly less effective in MR patients.

Mycoplasma pneumoniae is a common pathogen causing community-acquired respiratory tract infection mainly in children and young adults. Macrolides are generally considered to be the first-choice agents for treatment of *M. pneumoniae* infection. Although tetracyclines and fluoroquinolones are effective against *M. pneumoniae*, these agents are not recommended for children because of their toxicity. Tetracyclines can cause depression of bone growth, permanent gray-brown discoloration of the teeth, and enamel hypoplasia when given during tooth development. Although the clinical importance of fluoroquinolones has not been demonstrated, they produce cartilage erosion in young animals. Thus, these agents should be given only when there is no alternative (15).

As reported by Lucier et al. (9) and Okazaki et al. (14), an A-to-G transition or A-to-C transversion at position 2063 or 2064 of domain V of the *M. pneumoniae* 23S rRNA gene results in resistance to macrolide antibiotics. We have previously reported the isolation of macrolide-resistant (MR) *M. pneumoniae* from ca. 20% of clinical specimens collected from pediatric patients in Japan (11). Most of those isolates were highly resistant to 14-membered ring macrolides (MIC, >256 μg/ml) and moderately resistant to 15- and 16-membered ring macrolides.

Even in the cases of patients infected with MR *M. pneumoniae*, some pediatricians had the impression that there was a good response to macrolide therapy (11). There is a similar debate about the management of infection due to pneumococci. As noted in The Infectious Diseases Society of America (IDSA) guidelines for community-acquired pneumonia management (10), despite the increase of resistant isolates, a corresponding increase has not been seen in the number of clinical treatment failures.

One possible explanation for this is the nonantimicrobial effects of macrolides. It is known that macrolides have beneficial immunomodulating effects (1, 4, 6, 20), and they are clinically effective in hypersecretory conditions such as diffuse panbronchiolitis (7, 8) and cystic fibrosis (16). Thus, macrolides could be clinically effective even in MR *M. pneumoniae* infections.

It is important to know the clinical significance of MR *M. pneumoniae* infection, because in vitro susceptibility testing for *M. pneumoniae* is not available for daily management of patients. If macrolides are clinically effective against MR *M. pneumoniae* infection, pediatricians do not need to consider the use of tetracyclines or fluoroquinolones, even if the prevalence of MR *M. pneumoniae* rises in the future. Therefore, we performed a comparative study to determine whether or not MR *M. pneumoniae* influences the clinical outcome in patients treated with macrolides.

MATERIALS AND METHODS

Study population and sample collection. Three pediatric clinics in three different areas in Japan participated in this study. Sera and throat swabs or sputa taken from inpatients or outpatients suspected of *M. pneumoniae* infection were subjected to the laboratory tests.

Isolation. Isolation and identification of *M. pneumoniae* was carried out as described in a previous report (11).

PCR detection of *M. pneumoniae*. Sputa were obtained from patients, suspended in a small amount of saline, mixed well, and centrifuged at 2,000 rpm for 15 min, and then DNA was extracted from the supernatant with a QIAamp DNA Mini kit (QIAGEN K. K., Tokyo, Japan) according to the manufacturer's instructions. *M. pneumoniae* DNA was detected by the nested PCR method with primer sets for amplification of the P1 gene as previously described (17). The first

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Phone: (81) 425610771. Fax: (81) 425653315. E-mail: sasaki@nih.go.jp.

TABLE 1. Prevalence of macrolide-resistant *M. pneumoniae* in Japan

Year	Isolation method		DNA detection method	
	No. of isolates	No. of resistant isolates (%)	No. of specimens with positive *M. pneumoniae* DNA (no. of examined specimens)	No. of specimens with macrolide resistance mutation in *M. pneumoniae* DNA (%)
1999	296	0	12 (630)	0
2000	10	1 (10.0)	9 (92)	4 (44.4)
2001	6	2 (33.3)	28 (384)	3 (10.7)
2002	12	3 (25.0)	44 (352)	5 (11.4)
2003	54	7 (13.0)	10 (236)	1 (10.0)
2004	6	1 (16.7)	8 (183)	2 (25.0)
5-year total (2000–2004)	88	14 (15.9)	99 (1247)	15 (15.2)

primer set was ADH2F (5′-GGC AGT GGC AGT CAA CAA ACC ACG TAT-3′) and ADH2R (5′-GAA CTT AGC GCC AGC AAC TGC CAT-3′). The second primer set was ADH3F (5′-GAA CCG AAG CGG CTT TGA CCG CAT-3′) and ADH3R (5′-GTT GAC CAT GCC TGA GAA CAG TAA-3′).

Serological diagnosis. Particle agglutination (PA) antibody titers for *M. pneumoniae* were assayed by using Serodia-MYCO II (Fuji Rebio Ltd., Tokyo, Japan), which is manufactured using artificial gelatin particles, sensitized with cell membrane components of *M. pneumoniae*, according to the manufacturer's instructions.

Detection of resistance point mutations in domain V of 23S rRNA. MR *M. pneumoniae* isolates were screened on the basis of MIC of erythromycin (ERY), and identification of point mutations in domain V of 23S rRNA for ERY-resistant *M. pneumoniae* was performed according to our previously reported methods (11). For PCR-positive samples of *M. pneumoniae* DNA, the detection of a point mutation is indicative of a resistant phenotype because there is only a single rRNA operon in the genome (2). Neither plasmids with *erm* genes to mediate ribosomal modification nor any enzymes that inactivate macrolides have been found in *M. pneumoniae*. Thus, the prevalence of MR *M. pneumoniae* detected by the PCR methodology should reflect the true incidence of resistant strains.

Patient extraction for comparison of clinical courses. Clinical information was collected for the patients from whom *M. pneumoniae* had been isolated or its DNA detected. Patients who fulfilled the following criteria were extracted: (i) *M. pneumoniae* infection was laboratory confirmed, (ii) macrolides were prescribed during the illness, and (iii) complete information about prescribed antimicrobial agents and febrile days was available from the medical record. Laboratory-confirmed *M. pneumoniae* infection was defined as (1-a) isolation of *M. pneumoniae* from throat swabs or (1-b) detection of *M. pneumoniae* DNA from the sputum by PCR methods and serologically positive results, i.e., fourfold or greater rise of antibody titer in paired serum samples or titer higher than 1:640 in a single-serum sample by PA assay.

Patients infected with *M. pneumoniae* showing a point mutation in domain V of the 23S rRNA gene were defined as MR *M. pneumoniae*-infected patients (MR patients), and those infected with *M. pneumoniae* without the mutation were defined as macrolide-susceptible *M. pneumoniae*-infected patients (MS patients). MS patients were selected from the same study population as MR patients, and there were approximately twice as many of them as MR patients.

Measurement of clinical efficacy. To compare the clinical courses of MR and MS patients, we adopted the number of febrile days as a main outcome measurement. A febrile day was defined as a day during which the body temperature exceeded 38.0°C at least once. Total febrile days and the number of febrile days during macrolide administration were assessed. As these parameters would be affected by the time of commencement of macrolide administration, the number of febrile days before macrolide administration was also assessed. Other clinical symptoms and signs, such as cough and chest roentgenogram findings, were not taken into account in this study on account of the difficulty of objective and unified assessment through a retrospective review of medical records.

The numbers of patients whose prescribed antibiotic was changed were also compared. We speculated that a change in prescribed antimicrobial agent might reflect the pediatrician's clinical decision that the initial therapy had insufficient efficacy based on the general clinical condition of the patients. The pediatricians had no information about the susceptibility of *M. pneumoniae* at the time of clinical decision-making.

TABLE 2. Characteristics of enrolled patients

Characteristic	MR patients (n = 11)	MS patients (n = 26)	P
Age (yr)			
Median (range)	9.0 (0–13)	5.5 (1–14)	0.30
Mean	7.6	6.5	
Sex, male/female	4/7	14/12	0.33
No. of patients prescribed 14-membered ring macrolides (%)	8 (72.7)	7 (26.9)	0.025

Statistical analysis was performed using SPSS software, version 9.05 for Windows (SPSS, Inc., Chicago). Differences in categorical variables were assessed with the two-tailed Fisher's test, and for the comparison of medians the exact Wilcoxon rank-sum test was used. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Prevalence of MR *M. pneumoniae*. The prevalence of MR *M. pneumoniae* among clinical isolates and specimens with positive *M. pneumoniae* DNA is shown in Table 1. Before 1999, no MR *M. pneumoniae* was found among 296 clinical isolates. In 2000, however, MR *M. pneumoniae* appeared in 10% of isolates, and its prevalence rose to 33.3% in 2001. The overall prevalence of MR *M. pneumoniae* during 2000 to 2004 was 15.9%. All MR *M. pneumoniae* isolates had a resistance point mutation in domain V of 23S rRNA. A similar trend was seen in specimens with PCR-positive *M. pneumoniae*. Although the number of positive specimens before 1999 was limited (n = 12), no MR *M. pneumoniae* was detected. The prevalence of MR *M. pneumoniae* during 2000 to 2004, based on PCR-positive specimens, was 15.2%.

Comparison of the clinical courses between MR and MS patients. Eleven MR patients were selected for the analysis according to the criteria given above, and 26 MS patients were used as controls.

The patients' characteristics are summarized in Table 2. All patients were outpatients at the time of onset and had no severe underlying disease that might have influenced the clinical course. MR patients tended to be older and had a lower male/female ratio than MS patients, but the differences lacked statistical significance. Most patients were first prescribed β-lactam antimicrobial agents by primary physicians, followed by prescription of macrolides after attendance at a hospital. The prescribed macrolides differed among MR and MS patients. Significantly more MR patients than MS patients were prescribed 14-membered ring macrolides (72.7% versus 26.9%; P = 0.025). The majority of MS patients (19 out of 26 [73.1%]) were prescribed only 15-membered ring macrolides (azithromycin [AZM]).

The clinical courses in the MR and MS patients are summarized in Table 3. The total febrile days and the number of febrile days during macrolide administration were significantly greater in MR patients than in MS patients (median of 8 days versus 5 days [P = 0.019] and 3 days versus 1 day [P = 0.002], respectively). Febrile periods before macrolide administration, which consist of antimicrobial-free and mostly β-lactam-ad-

TABLE 3. Comparison of clinical courses in MR patients and MS patients

Characteristic	MR patients (n = 11)	MS patients (n = 26)	P
Febrile days			
Median (range)	8 (4–19)	5 (2–9)	0.019
Mean	9.3	5.5	
Febrile days during macrolide administration			
Median (range)	3 (1–11)	1 (1–5)	0.002
Mean	4.3	1.4	
Febrile days before macrolide administration			
Median (range)	3 (1–10)	4 (1–8)	0.402
Mean	3.8	4.1	
No. of patients with a febrile period exceeding 48 h after macrolide administration (%)	8 (72.7)	5 (19.2)	0.006
No. of patients with a change of prescription after macrolide administration (%)	7 (63.6)	1 (3.8)	<0.001

TABLE 4. Comparison of patients prescribed 14-membered ring macrolides

Characteristic	MR patients (n = 8)	MS patients (n = 7)	P
Total febrile days			
Median (range)	10.0 (4–19)	6.0 (4–9)	0.152
Mean	10.4	6.6	
Febrile days during macrolide administration			
Median (range)	3.5 (1–11)	1.0 (1–2)	0.004
Mean	4.9	1.1	
Febrile days before macrolide administration			
Median (range)	3.0 (1–10)	5.0 (3–8)	0.152
Mean	4.0	5.4	
No. of patients with a febrile period exceeding 48 h after macrolide administration (%)	7 (87.5)	1 (14.3)	0.01
No. of patients with a change of prescription after macrolide administration (%)	6 (75.0)	0	0.007

ministered days, showed no statistically significant difference (median of 3 days versus 4 days, $P = 0.402$).

The MR patients were more likely to have had the initially prescribed macrolide changed to another antimicrobial agent by their pediatricians (63.6% versus 3.8%; odds ratio, 43.8; $P < 0.001$). Among seven MR patients whose prescriptions were changed, all but one were changed to minocycline.

The results were similar for patients to whom 14-membered ring macrolides were administered (Table 4). Among these 15 patients (8 MR patients and 7 MS patients), 9 patients were prescribed clarithromycin, while the remaining 6 were prescribed ERY. Presumably due to the fact that the number of febrile days during macrolide administration was greater in MR patients than in MS patients (median of 3.5 days versus 1.0 day, $P = 0.004$), the initially prescribed macrolide was more frequently changed among MR patients than MS patients (75% versus 0%, $P = 0.007$). Although there was no statistical significance, there was a prolongation of total febrile days for MR patients (median of 10 days versus 6 days, $P = 0.152$).

When we focused on patients given 15-membered ring macrolides, 2 MR patients and 19 MS patients, the differences were not clear. Although there were only two MR patients in this group, their total febrile days and number of febrile days during macrolide administration were not different from those of MS patients (medians of 4.5 days versus 5.0 days and 1.0 day versus 1.0 day, respectively).

DISCUSSION

There are few reports on the isolation of MR *M. pneumoniae* from clinical specimens, and most of the isolates were obtained following ERY treatment (13, 19). In our survey, MR *M. pneumoniae* was not found in any of 296 clinical isolates or 12 *M. pneumoniae* PCR-positive specimens collected between 1983 and 1999, but it has been found in 15% to 20% of clinical isolates or PCR-positive specimens since 2000. MR *M. pneumoniae* first appeared in 2000 and rapidly spread throughout Japan (11, 12). Thus, it is important to evaluate the clinical significance of MR *M. pneumoniae*.

In our study, when patients infected with MR *M. pneumoniae* were treated with macrolides, the total febrile period was 3 days longer than that of patients with MS *M. pneumoniae*. Although we did not assess other clinical outcome variables, such as chest roentgenogram findings, a higher frequency of changes in prescription was observed in MR patients than in MS patients. This might reflect the pediatrician's judgment, based on the patient's clinical condition, that the initially prescribed macrolide was not sufficiently effective, even though the pediatricians had no information about the susceptibility of isolates at the time of clinical management. This tendency was also seen in patients who were treated only with 14-membered ring macrolides.

It was difficult to assess the immunomodulatory effects of macrolides in patients with *M. pneumoniae* infection in this study, because all the patients enrolled were prescribed macrolides according to the inclusion criteria. To evaluate the immunomodulatory effects of macrolides, it will be necessary to compare the clinical outcomes among MR patients treated with and without macrolides. An alternative is to compare the number of febrile days of MR patients with that of patients without antimicrobial agent therapy in the literature. According to review articles, fever might persist for about a week in the natural course of *M. pneumoniae* infection (3, 18). Kingston et al. (5) evaluated the effect of demethylchlortetracycline in a double-blind study, and the mean duration of fever in the treated group was 2.13 days, while it was 8.14 days in the placebo group. They started to count the number of febrile days not at the point of onset but only after entry into the study. In our study, the mean number of febrile days of MR patients was 9.2, which is similar to that of the placebo group

in Kingston's study. This implies that the antimicrobial effect is dominant over immunomodulatory effects in macrolide therapy, at least as far as duration of fever in *M. pneumoniae* infection is concerned. On the other hand, we did not assess the duration of other symptoms, such as malaise, sore throat, and cough, and it is possible that the immunomodulatory effects of macrolides can shorten these symptoms even in MR *M. pneumoniae* infection.

A difference of three febrile days in MR patients might not have a great impact in the management of *M. pneumoniae* infection, because it is often a mild and self-limiting disease, and the fever resolved even when the initially prescribed macrolide was not changed. However, it is reasonable to consider the use of alternative antimicrobial agents, such as minocycline, when macrolides are less effective than expected in patients more than 8 years old with possible *M. pneumoniae* infection.

The criteria for *M. pneumoniae* infection used in this study were stringent enough to confirm acute *M. pneumoniae* infection. This was a retrospective study based on a review of medical records, and patients with incomplete records were excluded. In general, clinical records of patients showing mild illness with *M. pneumoniae* infection were incomplete, and their clinical evaluation was excluded from this study.

In conclusion, we compared clinical outcomes in 11 MR patients and 26 MS patients given macrolide therapy. The MR patients showed more febrile days (by a median of 2 days) during the initial macrolide therapy than MS patients. On the other hand, no apparent treatment failure or serious illness was reported for MR patients. The influence of the emergence of MR *M. pneumoniae* on the treatment for *M. pneumoniae* infection deserves further study.

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Original Article

Epidemiological Studies on *Bartonella quintana* Infections among Homeless People in Tokyo, Japan

Naomi Seki[1,2]**, Toshinori Sasaki[2], Kyoko Sawabe[2], Tsuguo Sasaki[3], Mayumi Matsuoka[3], Yoshichika Arakawa[3], Eiji Marui[1] and Mutsuo Kobayashi[2]*

[1]*Department of Public Health, School of Medicine, Juntendo University, Tokyo 113-8421;*
[2]*Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo 162-8640; and*
[3]*Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011, Japan*

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SUMMARY: In an epidemiological investigation of trench fever in Japan, we compared the seroprevalence of *Bartonella quintana* in homeless people and in the general population. In homeless rescue outreach programs held in Tokyo from May 2001 to March 2003, 151 blood samples were taken from non-hospitalized homeless people. The prevalence of IgM and IgG antibodies against *B. quintana* in these people was compared with that in 200 healthy blood donors using a commercially available indirect fluorescent antibody test. Although IgG titers of > or = 1:128 were found in 57% (86/151) of homeless people and 51% (101/200) of blood donors, high titers of > or = to 1:1,024 were encountered only in homeless people (11%, 16/151). Attempts to isolate *B. quintana* from the blood of homeless people were unsuccessful, but polymerase chain reaction based detection, using *Bartonella* genus specific primers, demonstrated the presence of *B. quintana* DNA in the blood of 10 homeless people. Our data suggest that urban trench fever is endemic among the Japanese homeless population.

INTRODUCTION

The bacterium *Bartonella quintana* is the agent of trench fever, otherwise known as five-day fever, quitan fever and Wolhynia fever (27). Humans are the only known reservoir of *B. quintana*, and transmission among people occurs via the body louse (*Pediculus humanus*). The genus *Bartonella* now comprises 20 species or subspecies (15), several of which are recognised pathogens, including *B. henselae*, the agent of cat-scratch disease (CSD) (1), which is transmitted by cat fleas (*Ctenocephalides felis*) and dog fleas (*C. canis*) (30). Although trench fever has been recognised for almost a century, our perception of its medical importance has significantly changed in the last decade as new syndromes associated with the pathogen and new susceptible populations have been identified worldwide. Recently, there have been scientific reports of *B. quintana*-induced bacillary angiomatosis in patients from South Africa (4) and Zimbabwe (5), as well as widespread trench fever among Burundian refugees (21). In Europe and the United States, a broadening spectrum of *B. quintana* infections, ranging from life-threatening bacillary endocarditis to asymptomatic bacteraemia, are now being identified, particularly in homeless people (9), AIDS patients (2,12) and chronic alcoholics (16).

As in other industrialized countries, the size of the urban homeless population in Japan has increased markedly in recent years. In 2003, the Japanese Ministry of Health, Labour and Welfare estimated that the national homeless population had surpassed 25,000 (online only; available from: http://www.mhlw.go.jp/shingi/2003/12/s1216-5u.html) (18), and in the same year, official reports by local managers of parks, roads, and river basins in Tokyo's 23 cities indicated the presence of over 5,600 homeless people (29). Although *B. quintana* infections have yet to be documented in this population, the presence of *B. quintana* DNA in the body lice collected from several homeless volunteers provides strong support for their existence (23). In the study reported herein, we used serological and PCR-based methods to diagnose *B. quintana* in blood samples collected from homeless and normal populations in an attempt to clarify the epidemiological status of these infections in urban areas of Japan.

MATERIALS AND METHODS

Collection of blood samples: A prospective study was carried out in P city, an area inhabited by 250,000 people in the northwest of Tokyo that is characterized as a typical downtown area containing entertainment and business districts. An estimated 200 homeless people live around railroad stations and in the small parks scattered throughout the central downtown area. Since 1988, the P city municipal government has supported rescue outreach programs in which municipal officers provide occupational and medical consultations for these homeless people. The programs take place two to four times each year, and typically involve chest X-ray checks for pulmonary tuberculosis, provision of clothing and food, a haircut and a shower. Public health office doctors, nurses and sanitary inspectors provide infection control expertise and advice, specifically on tuberculosis and louse-born diseases.

The homeless people participating in these outreach programs were systematically approached for inclusion in the study, and the aims were clearly explained to each person approached. Prior to their inclusion in the study, informed

*Corresponding author: Mailing address: Department of Medical Entomology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2400, Fax: +81-3-5285-1178, E-mail: mutsuo@nih.go.jp

**Present address: Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK.

consent was obtained from all participants. The consenting participants underwent a brief clinical examination and were asked about their current health condition and any recent disease manifestations that they could remember. Blood samples were collected from an upper limb vein after iodine dressing. All participants were also asked if they had body louse or cat flea infestations. Public health nurses specifically checked participants for any clinical presentations associated with *B. quintana* infection.

Serological testing: For detection of specific antibodies to *B. quintana*, we used a commercially available indirect fluorescent antibody (IFA) kit (Focus Technologies, Cypress, Calif., USA). Control sera were chosen from healthy people who donated blood for the blood bank at the National Institute of Infectious Diseases, Tokyo. These controls were all males aged 30-60, and each group consisted of 50 samples.

Isolation and identification of *B. quintana* from blood samples: Approximately 3 ml of blood were inoculated into a 30-ml Bactec™ PEDS PLUSTM blood culture bottle (Dickinson & Company, Sparks, Md., USA) and incubated at 35°C for 7 days. An aliquot of each blood culture was then sub-cultured onto Columbia sheep blood agar plates and kept in a 5% CO_2 incubator for 3 weeks. A 500-μl aliquot of each blood culture was also prepared for use as template for polymerase chain reaction (PCR) by centrifugation at 15,000 rpm for 20 min, then DNA extraction by using the Sepa Gene kit (Sanko Chemicals, Tokyo, Japan). These extracts were incorporated into a *Bartonella*-specific nested PCR targeting a fragment of the 16S/23S rRNA intergenic spacer region incorporating the previously described primers QHVE-1/QHVE-3 (20) and our established *Bartonella* -specific nested primers QHVE-12 (5'-CCGGAGGGCTTGTAGCTCAG-3') and QHVE-14 (5'-CACAATTTCAATAGAAC-3'). The conditions used for both rounds of amplification were the same, and were comprised of one cycle of 94°C for 5 min, then 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, then one cycle of 72°C for 5 min. The PCR products were subjected to electrophoresis in 1% agarose gels, then visualized under UV illumination following ethidium bromide staining. These primers were also reacted with *B. henselae* DNA. The PCR products were then sequenced to identify *B. quintana* or *B. henselae*.

Statistical analysis: Statistical analysis was performed using statistical package SPSS for Windows, Release 10.0J (standard version; SPSS, Inc., Chicago, Ill., USA).

RESULTS

Demographics of the homeless population: Between May 2001 and March 2003, 232 people participated in 3 outreach programs, of whom 183 (79%) agreed to participate in our study by informed consent. From these cases, we selected 151 individuals for the study, all of whom were male and under 70 years old. We selected only men because we had only 2 female samples. If a person had been enrolled into more than one outreach program during the survey period, only samples and information gathered during their first attendance were used. Among the 151 homeless people included in the study, the mean age was 54.7 years (SD ± 7.7) and mean length of time spent homeless was 32 months (range 1 - 300 months, 2 unknown). The infestation rate with body lice (including those who had noted previous body lice infestation) was 11 % (16/151).

Serological analysis: The distribution of anti-*B. quintana* IgG titers in the homeless people and blood donors is presented in Figure 1. Titers of > or = 1:128 were detected in 86/151 (57%) of the homeless people but also in 101/200 (51%) of the blood donors. However, an IgG titer of 1:512 or greater was detected in 38/151 (25%) of the homeless people but in only 17/200 (9%) of the blood donors, and IgG titers of 1:1,024 or greater were detected only in the homeless people (16/151, 11%). The geometric mean IgG titers of the two groups were significantly different (the geometric mean in homeless and blood donors: 144 and 101, respectively, $t = 3.47$, $P < 0.001$). IgM titers below the cut-off titer should be negative, because significantly high levels of IgM antibodies were not detected (data not shown). Within the homeless population, variation in the prevalence of the serological evidence of *Bartonella* infection could only be correlated with the length of time spent homeless. Individuals who had been homeless for more than 1 year were significantly more likely to have serological evidence of infection than those who had been homeless for a shorter period (Wilcoxon Two-Sample test, $P < 0.05$) (Table 1).

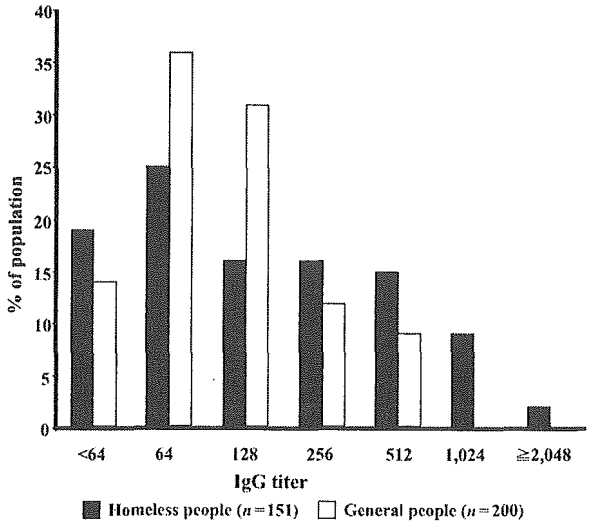

Fig. 1. Distribution of anti-*B. quintana* IgG antibody titers among surveyed homeless individuals and healthy blood donors.

Identification of *B. quintana* from blood samples: No isolates of *B. quintana* were obtained. However, we obtained PCR-based evidence of *B. quintana* infections in 10 individuals (7%, 10/151) (Table 1). The size of amplicons obtained using the PCR is dependent on the source of DNA, with different *Bartonella* spp. yielding different size products. Thus, the fact that all 10 amplicons were of an indistinguishable size of about 500 base pairs (bp) indicated that all products were probably derived from strains of *B. quintana* (Fig. 2). Other human-associated *Bartonella* spp. either failed to yielded an amplicon (*B. clarridgiae*) or yield an amplicon that was markedly larger (e.g., *B. henselae* 568 bp, *B. elizabethae* 572 bp)(data not shown). To confirm this, four of the amplicons were sequenced, yielding unambiguous sequence data for the entire length of the amplicon when primers QHVE12 and QHVE14 were used. Alignment and comparison of the sequences obtained demonstrated them to be indistinguishable from one another and from the ISR fragment previously determined for the *B. quintana* type strain (Fig. 2). Collation of serological data for the 10 PCR positive individuals revealed that six were seropositive (IgG titers more than 1:128) but four were seronegative (IgG titers less than 1:64).

Table 1. Comparison of demographic and diagnostic details of homeless individuals surveyed

	<64	64	128	256	512	≧1,024	Total (%)	*P* value
Age (yr)								
30s	0	2	4	0	0	0	6 (3.4)	
40s	4	11	4	5	4	3	31 (20.5)	n.s.
50s	14	18	13	8	7	7	67 (44.3)	
60s	10	6	3	11	11	6	47 (31.1)	
mean ± S.D. = 54.72 ± 7.7							151 (100.0)	
Period of streetlife (mo)								
<6m	14	17	8	5	6	3	53 (35.1)	
6m-1y	3	6	3	4	1	1	18 (11.9)	*P* < 0.01
1y-5y	8	10	8	8	11	8	53 (35.1)	
>5y	3	4	5	5	4	4	25 (16.6)	
unknown	0	0	0	2	0	0	2 (1.3)	
Mean ± S.D. = 32.56 ± 51.87 (range 1 - 300)							151 (100.0)	
PCR positive	3	1	1	3	2	0	10 (6.62)	n.s.

n.s. denotes not significant.

Table 2. Epidemiological and clinical findings associated with homeless individuals possessing high anti-*B. quintana* IgG titers

Symptoms	IgG titer ≧128 *n* = 86 *n* (%)			IgG titer ≦64 *n* = 65 *n* (%)			OR	95% CI
General fatigue	5 (5.8)			6 (9.2)			0.61	0.14 - 2.52
Fever	5 (5.8)			2 (3.1)			1.94	0.30 - 20.97
Headache	13 (15.0)			7 (11.0)			1.48	0.51 - 4.65
Anorexia	6 (7.0)			4 (6.2)			1.14	0.26 - 5.75
Night sweat	13 (15.0)			6 (9.2)			1.75	0.58 - 5.95
Arthralgia	23 (27.0)			16 (25.0)			1.12	0.50 - 2.53
Lymphadenopathy	2 (2.3)			2 (3.1)			0.75	0.05 - 10.63
Bone pain	7 (8.1)			8 (12.0)			0.63	0.18 - 2.13
Eruption	5 (5.8)			5 (7.7)			0.74	0.16 - 3.38
History of body lice infestation	11 (13.0)			5 (7.7)			1.76	0.53 - 6.80
History of fleas infestation	4 (4.7)			3 (4.6)			1.01	0.16 - 7.13
	Mean	Range	S.D.	Mean	Range	S.D.	*P* value	
Age (year)	55.35	34 - 68	7.76	53.89	31 - 68	7.59	0.2	
Period of street life (month)*	40.75	1 - 288	55.6	21.97	1 - 300	44.79	<0.01	

*seropositive population = 84 (2 unrecorded)

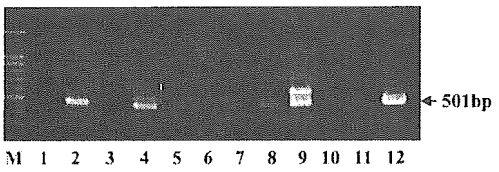

Fig. 2. Amplification products obtained from the cultured blood of Japanese homeless individuals by using a *Bartonella*-specific PCR targeting a fragment of the 16S-23S rRNA intergenic spacer region. M. marker. Lane 2, 4, 8, 9 show positive result. Lane12 is Fuccer strain of *Bartonella quinatata*.

None of the individuals with very high serological titers (>1,024) were found to be PCR positive.

Clinical characteristics: To evaluate if *B. quintana* seropositivity correlated with specific clinical features, we compared the results of clinical examinations of 65 seropositive homeless individuals with 86 seronegative individuals. Calculation of odds ratios (OR) and 95% confidence intervals (CI) indicated no significant differences between the two groups (Table 2).

DISCUSSION

We found clear evidence of *B. quintana* infections in a homeless population in an urban area of Japan using both serological and molecular methods. Comparison of our estimates of ongoing infections in about 7%, and past exposure in over 10% of a non-hospitalized homeless population with other studies is difficult, as there have been only several similar studies, and most of these have focused on hospitalized homeless populations. The principle reason behind this dearth of information is unlikely to be that infections are rare, but rather that they go unrecognized. To our knowledge, to date only Brouqui and colleagues have reported surveillance of non-hospitalized French homeless people for evidence of *B. quintana* infections (2). In this report, only 2% of 221 individuals tested had serological evidence of infection. Surveys of homeless people attending clinics or hospitals are more numerous and have yielded evidence of higher infection rates. Jackson and colleagues (12) reported 39 (20%) of 192 homeless attendees of a downtown clinic in Seattle had serological evidence of *B. quintana* infections, whereas Brouqui and colleagues (2) found that of 43 hospitalized