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RAPID AWARENESS AND TRANSMISSION OF SEVERE ACUTE RESPIRATORY SYNDROME IN HANOI FRENCH HOSPITAL, VIETNAM

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Abstract. A case-control study was conducted to examine the relationship between severe acute respiratory syndrome (SARS) and the time-dependent precautionary behaviors taken during an outbreak of SARS in Hanoi French Hospital (HFH), Vietnam. Masks (odds ratio [OR] = 0.3; 95% confidence interval [CI]: 0.1, 0.7) and gowns (OR = 0.2; 95% CI: 0.0, 0.8) appeared to prevent SARS transmission. The proportion of doctors and nurses who undertook each measure significantly improved (χ^2 = 9.8551, P = 0.043) after the onset of secondary cases. The impact of individual behaviors on an outbreak was investigated through mathematical approaches. The reproduction number decreased from 4.1 to 0.7 after notification. The basic reproduction number was estimated, and the use of masks alone was shown to be insufficient in containing an epidemic. Intuitive results obtained by means of stochastic individual-based simulations showed that rapid improvements in behavior and isolation would increase the probability of extinction.

INTRODUCTION

Notwithstanding the announcement of containment by the World Health Organization (WHO) in 2003,[1] severe acute respiratory syndrome (SARS) has remained a matter of concern worldwide, and it is not surprising that several cases of SARS have reemerged, for example, in China in April 2004.[2] Although the mode of transmission remains partially unclear, especially with regard to airborne transmission[3] and superspreading events,[4,5] it appears to occur predominantly by large droplets, direct contact with infectious material, or contact with fomites contaminated with infectious material.[6,7] The most effective containment measures identified to date include the tracing of contacts,[8] quarantine,[9] triage and early case detection,[10,11] and isolation.[12] Further, because the close contact required for transmission easily occurs in hospital settings,[13–15] nosocomial spread was determined as one of the major epidemiologic features of SARS.[7,16,17] The elimination of hospital transmission through enhanced infection control practices is therefore a crucial control measure.

An early study in Hong Kong showed that the practice of droplet and contact precautions was adequate in most clinical settings in significantly reducing the risk of infection after exposure to patients with SARS,[18] and if practiced by a high proportion of susceptible individuals, precautionary measures are expected to significantly reduce transmission.[19] The adoption of routine preventive behaviors based on appropriate training and control among health care workers (HCWs), undertaken prior to the isolation of SARS patients, was shown to be one of the most crucial control measures.[20–22]

In this context, Vietnam is considered to have achieved the first highly successful containment of SARS during the early phase of the outbreak.[23] One reason for this rapid containment is thought to be the prevention of infection leakage from hospitals back into the general community.[24] A second is the successful discontinuation of the chain of nosocomial transmission several days after onset based on the radical control measures of the Ministry of Health, Vietnam.[25] Although several nosocomial transmissions were observed in Hanoi French Hospital (HFH) in the early days of the outbreak,[26,27] none were identified in HFH or another local hospital in the latter phase.[28] In both hospitals, staff instituted stringent precautions, strict isolations, and quarantines under the encouragement of Dr. Carlo Urbani (Dr. Urbani died of SARS before seeing the success of the containment).[29] We therefore consider that a comprehensive understanding of the successful containment measures adopted by HFH and their theoretical underpinnings are crucial to the success of control strategies for any future recurrence. Here, we use a case-control study design to time-dependently examine the relationship between SARS and the precautionary behaviors undertaken by those exposed in HFH. We then use mathematical approaches to develop intuitive analyses of the impact of individual behaviors on the control of a SARS epidemic.

MATERIALS AND METHODS

Case-control study. HFH is a 56-bed secondary care hospital. After the admission of an index case on February 26, 2003, 38 cases in total were confirmed to have symptomatic SARS infection. The occurrence of newly diagnosed SARS cases due to local transmission continued until April 7, 2003, 3 weeks before the date when the Vietnamese government and WHO declared the outbreak successfully contained (April 28, 2003) (Table 1). The duration of the HFH outbreak was analyzed by separating it into three phases: Stage 1, February 26–March 4, from admission of the index case to the onset of secondary cases; Stage 2, March 5–March 10, from the suspicion of nosocomial spread to closure of the hospital; and Stage 3, from March 11 on, from strict isolation to local eradication.

A case-control study of 29 of the 38 laboratory-confirmed SARS cases and 98 controls was performed in HFH. The case group included 22 of 28 (78.6%) individuals admitted and retained in HFH and 7 of 10 (70.0%) individuals transferred to another hospital after first being admitted to HFH (total N = 29). The reasons for nonparticipation were death due to

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TABLE 1
Chronology of the outbreak of SARS in Hanoi French Hospital (HFH), Vietnam

Stage 1		
26-Feb-03	Day 0*	An index case complaining of fever, dry cough, and headaches was admitted to HFH.
2-Mar-03	Day 3	After intubation, the index case was isolated in ICU the following day.
4-Mar-03	Day 6	Nine secondary cases were suspected.
Stage 2		
5-Mar-03	Day 7	Seven additional cases were suspected. HFH informed the Ministry of Health, Vietnam, of the strange influenza. The health minister and experts from the World Health Organization (WHO) held a meeting. Dr. Carlo Urbani informed all staff to perform stringent precautions.
8-Mar-03	Day 10	HFH decided to close all outpatient/inpatient services. Visitors were not allowed to enter HFH. The hospital board of directors held an emergency meeting. Dr. Carlo Urbani explained the necessity of precautions and possibility of contamination as a mode of transmission. Health care workers were advised not to return home.
Stage 3		
11-Mar-03	Day 13	All inpatients were transferred to other hospitals. The 2nd floor of HFH was allocated to SARS patients only and strict isolation was enforced. • Three zones were allocated according to symptoms. • Nonmission individuals including health care workers were not allowed to enter.
13-Mar-03	Day 15	A special committee for SARS control and prevention was established. WHO issued a "global alert" to worldwide health authorities.
28-Apr-03	Day 60	The Vietnamese government and WHO declared successful containment of SARS in Vietnam.

* Day, days after onset of the outbreak. SARS, severe acute respiratory syndrome; ICU, intensive care unit.

SARS and/or respiratory failure (N = 5, 13.2%), refusal to take part (N = 1, 2.6%), or relocation (N = 3, 7.9%). The case group included 28 HFH employees (3 doctors, 13 nurses and nursing assistants, 10 radiologists and other co-medical workers, and 2 receptionist and administrative staff) and 1 relative of a patient. A further 23 Vietnamese patients who were directly admitted to another hospital were excluded because the detailed source of infection was unknown, although several cases were thought to have been infected in HFH. Detailed descriptions of the laboratory diagnoses were given previously.[28] They were confirmed through serological studies using an indirect enzyme-linked immunosorbent assay (ELISA) (Kirikae T, et al., unpublished data).

Controls were nominated based on employment in HFH and exposure among patients' relatives through HFH. The selection criteria included i) Vietnamese individuals more than 20 years old, ii) those who provided written informed consent based on explanation of our methods and purposes, and iii) those thought to have had contact with confirmed cases inside the hospital based on contact investigations. In total, 98 individuals were included as controls; most were HFH employees (13 doctors, 20 nurses and nursing assistants, 13 radiologists and other co-medical workers, and 11 receptionists and administrative staffs) or relatives of patients (N = 41). Although we investigated certain known contacts for inclusion as controls, namely individuals who took care of cases or entered cases' room, those who might have had trivial contact, such as possible exposure outside the hospital during, for example, transportation of SARS cases or in the casualty reception room, were not followed and included. The number of hospital employees investigated represented approximately 55.9% of the total employees used during the outbreak.

All participants were surveyed with regards to their use of personal protective equipment (PPE) and hygiene habits when in contact with patients with SARS; that is, the use of masks, gloves, and gowns, and the practice of hand washing, which were specifically recommended as droplet and contact precautions. In this paper, masks denote surgical masks; N95 masks were not available in the early stage of the outbreak in Vietnam. Individual behaviors were investigated mainly in two separate phases according to time-dependency (in Stage 1 and after entering Stage 2; i.e., Stages 2 and 3) (Table 1) to clarify any behavioral changes that occurred. Standardized questionnaires requiring one of two possible answers for each precaution ("performed" or "not performed") were given to each subject, and all responses were collected. Answers of "sometimes" or "seldom" were defined as "not performed" due to imperfect efficacy. In addition, the frequency of contact with infected individuals was investigated to represent the number of exposures per day. An exposure result of "many times" was recorded for those who had close contact with SARS patients, that is, those who cared for or lived with SARS patients, and those likely to have come into direct contact with the respiratory secretions or body fluids of SARS patients, for example, during close conversation (within 3 feet).[30] After completing the initial primary survey, an identical confirmation survey was performed to confirm the validity of the answers. These surveys were conducted along with other epidemiologic studies (Nishiyama A, et al., unpublished data) until mid-March 2004, almost 1 year after onset of the epidemic. No blood test results showing possible asymptomatic infections were available during the survey period. The participants were informed of how the information would be used and assured of the confidentiality of their responses. The purpose of the study was explained in Vietnamese, and written informed consent was obtained.

Statistical analyses were performed as follows. First, univariate associations between precautionary behaviors and infection were investigated in two separate stages (Stage 1 or Stages 2 and 3). Comparisons between groups were made using the χ^2 or Fisher's exact test for univariate analysis. Multivariate logistic regression was done in Stage 1 using forward stepwise selection (Waldesian) to determine the most significant variable associated with protection among those studied. Significant steps were taken to minimize recall bias with Stages 2 and 3 data. Analysis was restricted to those who had probable contact in these stages. It was further restricted to those cases developing symptoms whose incubation period

was within the greater than 95% confidence interval (95% CI) of having occurred after the beginning of Stage 2; and finally to medical doctors and nurses only, for both cases and controls. Second, univariate associations between sociodemographic variables (sex, age, and occupation) and SARS were investigated, with age and occupation categorized into four different groups each. Third, interactions between the identified most significant protective behavior and other variables significantly associated in univariate analysis were investigated through the use of crosstabs statistics, in which the odds of being infected were stratified according to a comparison of variables, and interactions were sought through the different odds ratio in each strata. Finally, multiple logistic regression analysis was used to determine the protective effect and eliminate confounding variables. As described in the next section, all variables significantly associated in univariate analyses, as well as sociodemographic variables, were selected and entered together in the final model. All data were entered into Microsoft Excel 2000 (Microsoft Co., Redmond, WA), and the statistical data were analyzed using the statistical software "R" (R Development Core Team, Vienna).[31]

Mathematical methods. The predictive effects of the behavioral changes were simulated using an individual-based stochastic model. For ease of understanding, a compartmental model, a type of SEIR (susceptible [S], exposed [E], infected [I], and recovered/removed [R]) model, which considered the process of transmission according to the protective behaviors taken against infectious contact among susceptible individuals, was applied. Instead of assuming "exposed (latent)" and "infectious" periods, E and I were defined as "incubation" and "symptomatic" periods, respectively, as the infectious period of SARS has not been fully clarified. Although SEIR models are usually deterministic and use mean estimations as model parameters, even with regard to SARS,[12,32] stochastic simulations were performed in this study because of the need to consider the stochasticity of each protective behavior, and also because of the small sample population size. The infectious lifetime of each individual was presented as an absorbing Markov chain. The simulations start with an individual index case (Day 0) in a population of 300 in which all individuals are susceptible.

Of the total 127 subjects studied (29 cases and 98 controls), 62.2% (N = 79) were considered to have had casual contact and 37.8% (N = 48) to have had close contact with SARS patients. The number of casual contacts (κ_1) was directly obtained (= 0.7 ± 0.2 [day^{-1}]), while the mean of close contacts (κ_2 = 0.4 [day^{-1}]) was determined with the following equation:

$$\kappa_2 = \kappa_1 \ln(\mathrm{OR}_{closed}) \quad (1)$$

where OR_{closed} (= 2.5; 95% CI: 1.1–5.9) denotes the odds ratio (OR) of getting infected as a result of close contact. In other words, to quantify close contact, we assumed that the frequency of infection is mainly determined by the frequency of contact, so that the ratio of the frequency of close to casual contact becomes proportional to the logarithm of the OR of transmission. The protective effect of precautionary behavior was approximated by:

$$\beta = 1 - \mathrm{RR} = 1 - \frac{a(c+d)}{c(a+b)} \approx 1 - \frac{ad}{bc} \approx 1 - \mathrm{OR} \quad (2)$$

where RR and OR denote the relative risk and odds ratio, respectively, of becoming infected while performing a protective behavior (with precaution = with exposure). Here, a is the number of exposed ill people; b, the number of exposed healthy people; c, the number of unexposed ill people; and d, the number of unexposed healthy people. If the outcome (i.e., disease investigated) is a rare event, that is, if a and c are very small compared with b and d, respectively, $(a + b)$ and $(c + d)$, respectively, would be closely similar to b and d alone. In this case, OR would approximate RR.

The lengths of the incubation and symptomatic periods were both assumed to be independently and identically distributed random variables with a probability density function of γ distribution, the mean and variance of which were defined as 3.8 [days] and 8.3 [days^2], and 16.2 [days] and 7.9 [days^2], respectively.[24,33] These distributions were applied to difference equations (as a discrete time model) by discretizing the probability density functions by day (for a detailed description of the simulation algorithm, see the Appendix).

The first simulation scenario hypothetically investigated the unchanged coverage and mean protective effects of a behavioral measure throughout the epidemic. Primary information on protective behaviors was obtained from our Stage 1 survey. Estimates for the extent of a protective effect, the associated causative behavior of which was found in forward stepwise logistic regression to be the most significantly associated with protection (as described above), were obtained through the use of further multivariate logistic regression analysis. This analysis incorporated all variables significantly associated with SARS on univariate analysis (i.e., other precautionary behavior, gender, age and occupation). To investigate the impact of the coverage of a protective measure on the trajectory of an outbreak, sensitivity of the cumulative number of SARS cases at Day 30 to the coverage of masks was investigated in the mean field equation. In the second scenario, it was assumed that coverage improved dramatically after entering Stage 2 (Day 7) due to an awareness of transmission. Further, in Stage 3 (Day 13), the hospital implemented not only stringent precautions but also strict isolations. To understand the trajectory of transmission in detail, the number of incubating as well as symptomatic individuals was investigated. As was in fact seen during Stage 3 of the outbreak, it was also assumed that all cases who became symptomatic were immediately isolated and that nobody except a limited number of healthcare workers were permitted to have contact with them. Because the greatest uncertainty applies to the time taken to increase coverage of a protective measure and to implement strict isolations, sensitivity analyses comparing the cumulative number of SARS cases up to Day 30 were performed with the time to change both protective measures set simultaneously on the same day. Finally, the basic reproduction number was estimated using the (effective) reproduction number obtained in Stage 1 (see Appendix).

RESULTS

Table 2 shows the univariate association between the precautionary behaviors taken (SARS and non-SARS [control] cases) in Stage 1 and SARS. The use of masks (P = 0.011) and gowns (P = 0.012) appeared to prevent infection, whereas handwashing and the use of gloves were less likely to provide protection. Only two subjects who performed all pro-

TABLE 2
Precautionary measures taken by all participants in Stage 1

	SARS cases (N = 25)	Non-SARS (N = 90)	P value*	Odds ratio† (95% CI)‡
All measures	2	44	0.059	0.2 (0.0–1.0)
Handwashing before§	12	51	0.937	1.0 (0.4–2.3)
Handwashing after¶	15	56	0.766	1.1 (0.5–2.8)
Masks‖	8	35	0.011	0.3 (0.1–0.7)
Gloves	8	30	0.643	0.7 (0.3–1.9)
Gowns	2	25	0.012	0.2 (0.0–0.8)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

tective measures developed symptomatic infections (P = 0.059). Forward stepwise logistic regression of the five protective measures (0.05 for entry and 0.10 for removal probability) showed that only the use of masks was significant in the final model (OR, 0.29, 95% CI; 0.11-0.73, P = 0.009). In Stages 2 and 3, the use of masks (P = 0.001) and gowns (P = 0.010) was significantly associated with non-infection among doctors and nurses still not infected after Stage 1 (Table 3). Most performed all the personal protective measures recommended, and only one individual who wore masks was infected. The comparative results of the behaviors of all participants at Stage 1 and after entering Stage 2 are shown in Figure 1a. The proportions of individuals who performed the investigated protective behaviors increased after entering Stage 2. However, these behavioral changes were not significantly different between the two phases (P = 0.960). The behaviors performed by the doctors and nurses (N = 48; Figure 1b) who had the closest contact with the SARS patients drastically and significantly improved after entering Stage 2 (χ^2 = 9.855, P = 0.043).

The univariate associations between socio-demographic variables and SARS throughout the epidemic are shown in Table 4. Females were more likely to become infected than males (P = 0.011), and a significant association of SARS with nurses (P = 0.008) was observed. In HFH, infection was frequent in the 40–49 age strata (P = 0.015). Among all study subject, relatives of patients (P < 0.001) appeared to be the least frequently infected. Table 5 shows the interaction between the use of masks and other significantly associated variables in univariate analyses. Even though we saw no significant difference in the OR of using masks versus the use of gowns, females (OR = 0.2) and nurses (OR = 0.1) were more effectively protected by the use of masks than others in Stage 1. In Stages 2 and 3, the use of gowns showed overall reasonable OR (= 0.2), whereas most other interactions could not be calculated due to the scarcity of cases.

TABLE 3
Precautionary measures taken by health care workers in Stages 2 and 3

	SARS cases (N = 4)	Non-SARS (N = 26)	P value*	Odds ratio† (95% CI)‡
All measures	1	25	0.001	< 0.1 (0.0–0.3)
Handwashing before§	4	25	1.000	NC
Handwashing after¶	4	25	1.000	NC
Masks‖	1	25	0.001	< 0.1 (0.0–0.3)
Gloves	4	25	1.000	NC
Gowns	3	26	0.010	NC

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

Figure 2a shows the mean and corresponding 95% CI of the trajectory (shown as prevalence) of an epidemic from 250 simulation runs which hypothetically assumed unchanged coverage as well as the protective effects of the precautionary measures observed in Stage 1. The precautionary measure in this simulation was based on a multivariate logistic regression which included all variables showing significant associations in univariate analyses, and focused on the impact of the use of masks, given the identification of this behavior as the most important protective measure (β = 0.6 obtained from OR = 0.4, P = 0.020). The coverage of masks was obtained as 52.0% from Table 2. If an outbreak was simply allowed to continue growing under these conditions, the results showed that approximately 50 to 90 symptomatic cases would occur by Day 30. The reproduction number (R) was estimated as 4.1 (95% CI; 1.9–6.4), and from this estimate the basic reproduction number was estimated as 6.0. Sensitivity of the cumulative number of cases to the coverage of masks, in the mean field, is shown in Figure 2b. Certain reduction in the cumulative number of cases was observed with significant improvements in coverage.

Figures 2c and 2d shows the outbreak trajectory of 250 simulations assuming improved coverage (from 52.0 to 81.5%) among susceptible individuals on Day 8 and restriction of contact with symptomatic individuals to health care workers on Day 13. The protective effect obtained from multivariage regression was 0.9 (OR = 0.1, P = 0.955). The reproduction number in Stage 2 was estimated as 0.7 (95% CI; 0.0–2.3). The number of incubating individuals began to show a decreasing trend after these events (Figure 2c), followed by a declining trend in the number of symptomatic cases (Figure 2d). Most of the simulated outbreaks eventually declined to extinction before Day 120. The sensitivity of the final size of an epidemic, evaluated through observations of the cumulative numbers of cases, to the timing of drastic changes in protective behaviors accompanied by strict isolation is shown in Figure 2e. When the stochastic effects are taken into account together with the effects of single precautionary measures and isolation, the rapid implementation of combined measures reduces the number of transmissions and increases the probability of extinction.

DISCUSSION

The findings of this case-control study indicate that the use of masks was significantly associated with the prevention of SARS transmission and that precautions against droplet contamination and contact were adequate in preventing transmission; this implies mainly to in-hospitals. The results are roughly consistent with those of previous reports.[18,20,22] Although a number of exceptions were seen with regard to protective effects during patient intubation, during which transmission to staff occurred even when droplet and contact precautions were taken,[7,34] one of the most important lessons from the SARS outbreak is the need to enhance infection control programs in hospitals.[13,35] Even though the use of

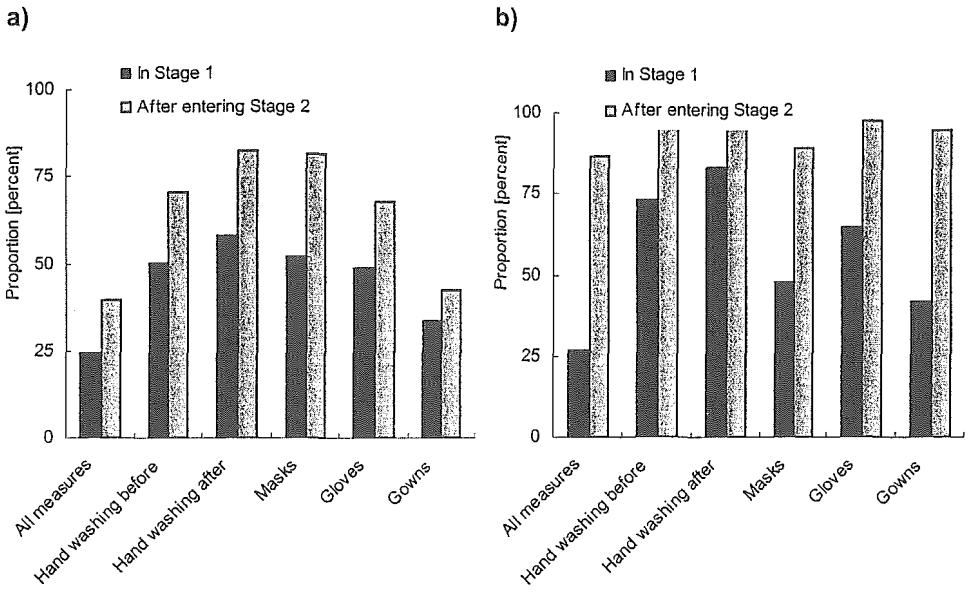

FIGURE 1. Protective behavioral changes defined by stage. **a**, Proportion of participants (SARS and non-SARS [control] cases) who performed each precautionary measure in Stage 1 (N = 127) and after entering Stage 2 (N = 108). Handwashing "before" and "after" denote before and after contact with a patient, respectively. **b**, Proportion of health care workers who performed each precautionary measure in Stage 1 (N = 48) and after entering Stage 2 (N = 37).

masks was the most effective precautionary measure, masks alone together with the observed coverage did not reduce the reproduction number below unity (R_0 = 6.0 and R with the protective effects of masks = 4.1). Put simply, the use of masks alone was shown to be insufficient to contain the epidemic. Further, it was shown that the coverage of precautionary behaviors among the study subjects increased with the progression of the outbreak, and this was especially obvious among doctors and nurses. In HFH, remarkable changes occurred in the very early phase of the outbreak before detailed information about SARS was available. According to the stochastic simulations, an increased probability of extinction would be observed if the combined measures of precaution and isolation were rapidly implemented.

With regard to sociodemographic variables, females were more frequently infected than males. Given that transmission was most frequently observed among nurses, a plausible explanation for this finding would be occupational background. Although the 40–49 age group was frequently infected, we have no persuasive explanation for this apart from occupation: 61.9% of this stratum was medical doctors or nurses. Considering that nurses were more effectively protected from transmission by the use of masks, the control measures taken by them within HFH from early in the epidemic were admirable. The lowest frequency of infection was seen in relatives of patients, showing that our study included many relatives who remained uninfected but were nevertheless believed to have had contact. Because nonmatched case-control designs such as this are vulnerable to selection bias, we obtained estimates of the protective effect of masks by means of multivariate logistic regression analysis which entered all other variables significantly associated with infection in univariate analysis. After adjustment for internal confounding variables, the estimated reproduction number was given as 0.7 in Stages 2 and 3. Previous studies have shown that the (effective) reproduction number, defined as the average number of secondary cases generated by one index case in a susceptible population under certain restrictions and interventions, decreases with increasing awareness of the epidemic combined with several public health measures.[36,37] Using reasonable estimation procedures, another study showed that R significantly decreased after a global alert in most affected countries.[38] The current study showed that the estimated R decreased below unity after notification of a hospital outbreak, although the estimates were obtained using rough assumptions and the process of estimation was biased by various factors.

In HFH, the rapid increase in awareness, which led to not only strengthened precautionary measures and isolation but also quarantining of health care workers, seems to have been the greatest contributor to successful containment. One reason for this quick response could be attributed to the background of secondary cases that arose mainly from health care workers who had close contact with the index case. Almost all staff members working or on duty in the earliest days of the

TABLE 4
Univariate associations between age-class/occupational categories and SARS

	Category	N	P value*	Odds ratio (95% CI)†
Sex	Male	47	0.011	0.3 (0.1–0.8)
	Female	70	0.011	3.3 (1.2–9.0)
Age class	29 y/o	29	1.000	0.9 (0.3–2.3)
	30–39 y/o	44	0.080	0.4 (0.2–1.1)
	40–49 y/o	42	0.015	2.8 (1.2–6.6)
	50 y/o	12	0.733	0.7 (0.1–3.2)
Occupation	Medical doctors	16	1.000	0.8 (0.2–2.9)
	Nurses	33	0.008	3.2 (1.3–7.7)
	Other co-medicals	36	0.076	2.2 (0.9–5.2)
	Relatives of patients	42	< 0.001	< 0.1 (0.0–0.4)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.

TABLE 5
Interactions between wearing masks and other variables on the infection

	In stage 1			In stages 2 and 3		
	Odds for masks (+)	Odds for masks (−)	Odds ratio*	Odds for masks (+)	Odds for masks (−)	Odds ratio*
Gowns						
(+)	0.3	0.6	0.5	< 0.1	2.0	0.2
(−)	0.3	0.5	0.6	NC	NC	NC
Sex						
(male)	0.1	0.2	1.0	0.0	0.0	NC
(female)	0.2	0.8	0.2	0.1	NC	NC
Age class						
29 y/o	0.1	0.4	0.3	0.0	NC	NC
30–39 y/o	0.1	0.3	0.5	0.0	NC	NC
40–49 y/o	0.3	0.8	0.3	0.1	1.0	0.1
50 y/o	0.2	0.2	1.0	0.0	NC	NC
Occupation						
(Medical doctors)	NC	0.6	NC	0.0	0.0	NC
(Nurses)	0.2	1.6	0.1	0.1	0.0	NC
(Other co-medicals)	0.5	0.5	1.2			
(Relatives of patients)	NC	0.1	NC			

NC = not calculable.
* Odds ratio of being infected while taking specific precautions.

outbreak (in Stage 1) were severely infected.[39,40] Another reason might be due to the efforts led mainly by Dr. Carlo Urbani, who suggested quick improvements in the precautionary measures taken and isolation.[29] As a result, transmission leakage into the community was prevented, thus having a huge impact on the chains of transmission.[24] In HFH, those who were exposed implemented precautionary and other controlling measures quickly and efficiently, and the epidemic consequently declined to extinction.

In the interests of objective interpretation, the limitations of our study design must be addressed, as follows:

1) A study such as ours in which exposure has a strong intuitive causal link with outcome (i.e., mask usage) is vulnerable to recall bias. Even though we limited our subjects in Stages 2 and 3 to medical doctors and nurses, and cases were appropriately selected according to the probable date of infection and incubation period, our estimates are likely less accurate than would be obtained by blinded or matched case-control study. In addition to this directional bias, further bias may have been introduced by random misclassification, as our records were completed 1 year after the outbreak, and it is therefore possible that some of the precautions were uncertain exposures. The frequent use of masks among controls may have reduced the strength of the associations.
2) Model-generated results must be interpreted cautiously. Although the simulations shown here included only the effect of masks and were considered according to the results of multivariate logistic regression adjusted for internal factors, unknown external confounding factors likely exist. For example, in Stages 2 and 3, although multivariate logistic regression was performed with other variables, the P value obtained was 0.955, and overall the model was weak. Owing to the scarcity of case records, stratification in this stage failed to separate the effects of masks. Thus, the estimates of the protective effect of masks and reproduction number in this stage may include the effects of other concomitant changes, such as the reduced frequency of contacts and quarantine.
3) There are limitations concerning the simplicity of our model; for example, we neglected the possible differential susceptibility of humans to asymptomatic infections,[41,42] individual variance in severity and/or prognosis,[23,43,44] and the highly heterogenous transmission of SARS.[4,5,45] Theoretical exercises never replace reality.
4) Finally, because our model was based on a case-control study, the estimates of coverage were biased; principally, coverage in a case-control design is taken from a nonrepresentative sample. Although this study was conducted as a first attempt to incorporate the effect of behavioral factors, which change time-dependently, to model building strategies for the control of directly transmitted airborne diseases, further studies incorporating a number of methodological improvements are required.

In conclusion, given that early recognition that leads to the implementation of protective behaviors and effective control strategies is crucial in hospitals,[46] we believe our model provides intuitive results that at least partly satisfy the need to evaluate outbreak trajectories based on individual behaviors.

APPENDIX

Each simulation starts with one index case and is based on a model constructed as follows:

i) The expected number of people who used protection on each subsequent day was determined by the number of susceptible individuals (S), number of contacts per day (κ), proportion of individuals who performed the protective behavior (p), and the protective effect of the precautionary measure (β), which were obtained based on our survey. The number of infectious contacts, denoted by the product of the number of susceptible individuals (S) and the mean number of contacts (κ), was divided into two subgroups: one that represents protection due to precautionary behaviors against infection with SARS-CoV (SARS-associated coronavirus) and another that does

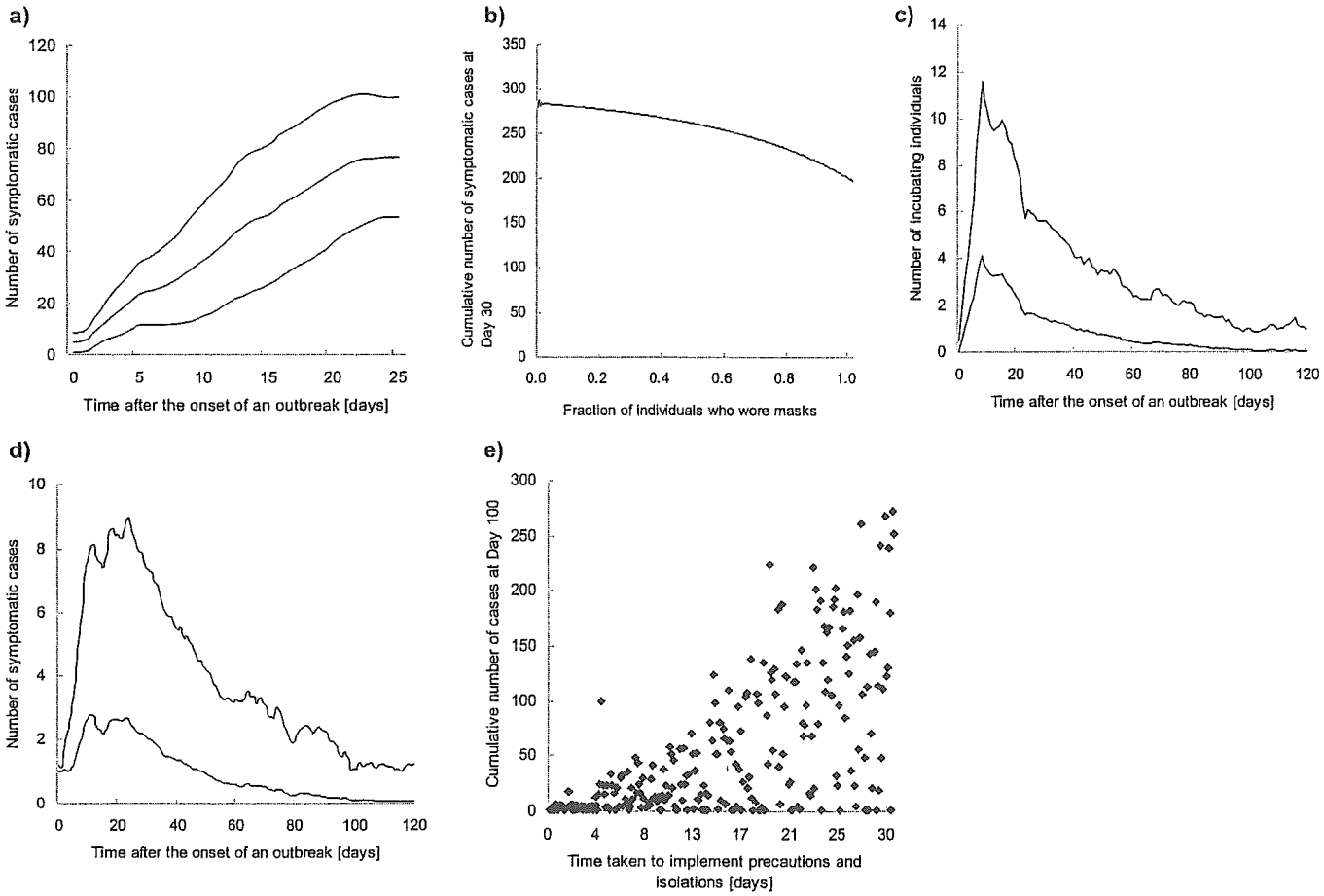

FIGURE 2. Stochastic simulations of a SARS outbreak with dependency on the coverage and protective effect of precautionary behaviors. **a**, Predicted number of symptomatic cases and corresponding 95% confidence interval (95% CI) given by 250 simulation runs assuming unchanged (stable) protective behaviors for the entire period. The reproduction number (R) was 4.1 ± 1.1. **b**, Sensitivity of the cumulative number of cases at Day 30 to the coverage of masks. The obtained line represents the simulation based on mean field (without assuming random function with binomial distribution in each transition probability). The protective effect of wearing a mask was fixed ($\beta = 0.6$). **c** and **d**, Stochastic simulations of a SARS outbreak with dependency on a combination of precautionary measures and strict isolation. **c**, The mean number of incubating individuals and corresponding 95% CI from 250 runs with changes in protective behaviors combined with strict isolation (lower 95% CI is x-axis). At Day 7, the effectiveness/coverage of precautionary measures used improved from 0.6/52.0 to 0.9/89.2, respectively. At Day 13, the number of susceptible individuals decreased from 300 to 20. The reproduction number decreased from 4.1–0.7 ± 1.1–0.8. **d**, The mean ± 95% CI of symptomatic cases given by 250 runs assuming changes in protective behaviors combined with strict isolation. The conditions were the same as those in **c**. **e**, Sensitivity of the size of an outbreak (represented by the cumulative number of cases) to the time taken to enhance precautionary measures and implement strict isolation; the combined measures are started at the same time and under the same conditions as in **c**.

not, according to $(1 - p\beta)$. However, these groups were not permanently fixed. The mean of the number of contacts based on our survey was approximated by:

$$\kappa = \kappa_1\pi_1 + \kappa_2\pi_2 = \kappa_1\pi_1 + \kappa_1 \ln(\mathrm{OR}_{\mathrm{close}})\pi_2 \qquad \text{(A1)}$$

where κ_1, κ_2, π_1, and π_2 denote the number of casual and close contacts and the fraction of individuals who had casual and close contacts, respectively, while the odds ratio of getting infected with close contact is represented by $\mathrm{OR}_{\mathrm{close}}$ and N, respectively.

ii) Both the incubation (E) and symptomatic (I) periods were assumed to be independently and identically distributed following an approximated probability density function with gamma distributions[33] (denoted by γ_k and c_l for the discretized stages [days] k and l, respectively). We divided the probability density functions into k ($i = 14$) and l ($j = 12$) stages; the methodology of approximation by date was previously reported.[24] The relative measure of infectiousness for the incubation (E) period (q) was assumed to be 0.1.[12]

iii) Based on realistic settings in Vietnam, it was assumed that all individuals were isolated with the onset of early signs of clinical symptoms under the isolation measures; and for simplicity, the effect of quarantine was neglected. When considering strict isolation, the number of susceptible individuals having contact with SARS patients was limited to 20 (which is the approximate number of ward workers); the number of susceptible individuals was treated as being stable (always $S = 20$) so that S would not be exhausted thereafter; without isolation there were assumed to be 300 susceptible individuals (which is roughly the total number of people involved in possible contacts in HFH). $N = S + E + I + R$, and background mortality was neglected. The resulting simplest difference equations were formulated as follows:

$$
\begin{aligned}
S(t+1) &= \exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]S(t) \\
E_1(t+1) &= \left\{1-\exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]\right\}S(t) \\
E_k(t+1) &= (1-\gamma_{k-1})E_{k-1}(t) \qquad \text{(A2)} \\
I_1(t+1) &= \sum_{k=1}^{i}\gamma_k E_k(t) \\
I_l(t+1) &= (1-c_{l-1})I_{l-1}(t) \\
R(t+1) &= R(t) + \sum_{l=1}^{j} c_l I_l(t)
\end{aligned}
$$

Based on the forward stepwise logistic regression result in the case-control study, and to facilitate understanding, p and β were used only to represent the use of masks. However, the protective effect, β, was obtained from the result of further multiple logistic regression which entered all other significantly associated variables (in univariate analysis). All terms shown here as products of a probability and a state variable were generated in our simulations by using random variables with binomial distributions. Under these assumptions and using mean length of incubation and symptomatic periods, the reproduction number (R) is given by:

$$R = \kappa(1-p\beta)\left(\frac{q}{\gamma}+\frac{1}{c}\right) \qquad \text{(A3)}$$

where γ^{-1} and c^{-1} are the means of the incubation and symptomatic periods in days, respectively. The basic reproduction number was estimated by

$$R_0 = \frac{R}{(1-p\beta)} \qquad \text{(A4)}$$

For the purpose of mathematical convenience, although unrealistic, our model assumed homogenous mixing as well as all infectious individuals being equally infectious.

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Rapid and Simple Method for Detecting the Toxin B Gene of *Clostridium difficile* in Stool Specimens by Loop-Mediated Isothermal Amplification

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We applied the loop-mediated isothermal amplification (LAMP) assay to the detection of the toxin B gene (*tcdB*) of *Clostridium difficile* for identification of toxin B (TcdB)-positive *C. difficile* strains and detection of *tcdB* in stool specimens. *tcdB* was detected in all toxin A (TcdA)-positive, TcdB-positive (A^+B^+) and TcdA-negative, TcdB-positive (A^-B^+) *C. difficile* strains but not from TcdA-negative, TcdB-negative strains. Of the 74 stool specimens examined, A^+B^+ or A^-B^+ *C. difficile* was recovered from 39 specimens, of which 38 specimens were LAMP positive and one was negative. Amplification was obtained in 10 specimens that were culture negative, indicating that LAMP is highly sensitive. The LAMP assay was applied to detection of *tcdB* in DNA extracted by a simple boiling method from 47 of those 74 specimens, which were cultured overnight in cooked-meat medium (CMM). Twenty-two of 24 culture-positive specimens were positive for LAMP on DNA from the culture in CMM. Four specimens were culture negative but positive by LAMP on DNA from CMM cultures. The LAMP assay is a reliable tool for identification of TcdB-positive *C. difficile* as well as for direct detection of *tcdB* in stool specimens with high sensitivity. Detection of *tcdB* by LAMP from overnight cultures in CMM could be an alternative method of diagnostic testing at clinical laboratories without special apparatus.

Clostridium difficile is well known as a cause of pseudomembranous colitis and a principle causative agent of antibiotic-associated diarrhea. Rapid and sensitive laboratory diagnostic testing is highly desirable for appropriate treatment of *C. difficile*-associated diarrhea (26). Two toxins, toxin A (TcdA) and toxin B (TcdB), are involved in the pathogenicity of this organism. The cell culture assay with the neutralization test is still used as a sensitive and specific method to detect TcdB, although the method is not easy to perform, cost-effective, or highly standardized.

A number of commercial tests are available for rapid and simple immunological detection of TcdA alone or of both TcdA and TcdB. However, these tests were found not to be as sensitive as the cell culture assay (6, 19, 22), and the infection caused by TcdA-negative, TcdB-positive (A^-B^+) *C. difficile* could not be diagnosed by using only the TcdA detection kit (1, 3, 16, 17, 18). Culture of *C. difficile* is a sensitive and specific method when cultured isolates are tested for TcdA and TcdB production (7). The PCR assay for detecting the toxin genes has been widely used for identification of types of toxin produced by recovered isolates (3, 12, 14, 27). Detection of *tcdA* and/or *tcdB* in stool specimens by PCR (9, 15), nested PCR (2), and real-time PCR (5) has also been developed and evaluated. Although reported to be rapid and sensitive diagnostic methods, they are not necessarily of practical use in clinical laboratories, where special equipment such as a thermal cycler or detection systems are not available.

Recently, loop-mediated isothermal amplification (LAMP) has been developed as a novel method that amplifies DNA with high specificity and simplicity (20, 21). In this study, we evaluated a LAMP method for identification of TcdB production by recovered isolates as well as for direct detection of *tcdB* in fecal specimens. DNA extraction from stool specimens requires some tedious steps to remove amplification inhibitors, making it difficult to do routinely in clinical laboratories. To further simplify the methods, the LAMP method was applied to detection of *tcdB* in DNA extracted by a simple and quick boiling method from stool specimens which were cultured overnight in cooked meat medium (CMM).

MATERIALS AND METHODS

Bacterial strains. The 40 *Clostridium difficile* strains used in this study were clinically isolated at various hospitals in Japan and previously classified into toxinotypes (Table 1) (24). Strains of *Clostridium* species other than *C. difficile* were obtained from the Japan Collection of Microorganisms except for an enterotoxin-positive *Clostridium perfringens* strain, MRY 05-0166, which was recovered from a case of antibiotic-associated colitis (Table 1).

Stool specimens. Stool specimens were obtained with the informed consent of patients admitted to five hospitals in Japan, who were given the diagnosis of antibiotic-associated diarrhea or colitis. The stool specimens were frozen at −80°C until transported and tested at the National Institute of Infectious Diseases. All tests were performed in batches on the same day, after a single thawing of the stored specimen.

Culture. *C. difficile* was isolated on cycloserine-cefoxitin-mannitol agar (CCMA) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) from stool specimens, which were treated with alcohol for spore selection. *C. difficile* was identified by colony morphology on CCMA and cell morphology after Gram staining. The latex agglutination test detecting glutamate dehydrogenase (Shionogi Pharmaceutical Co., Ltd., Tokyo, Japan) was used to confirm the identification.

The nonrepeating and repeating sequences of *tcdA* were amplified by PCR with primer sets NK3-NK2 (14) and NK9-NK11-NKV011 (11, 12), respectively. The presence of *tcdB* was examined by PCR with primer set NK104 and NK105 (12).

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TABLE 1. Strains used in this study and results of detection of *tcdB* by PCR and LAMP

Species (strain no.)	Tcd type	Toxinotype[a]	Detection[b] of *tcdB* by:		No. of strains studied
			PCR	LAMP	
Clostridium difficile	A^+B^+	0	+	+	20
	A^+B^+	I	+	+	1
	A^+B^+	III	+	+	1
	A^+B^+	IV	+	+	1
	A^+B^+	IX	+	+	1
	A^+B^+	XII	+	+	1
	A^+B^+	XVIII	+	+	1
	A^+B^+	XIX	+	+	1
	A^+B^+	XX	+	+	1
	A^-B^+	VIII	+	+	5
	A^-B^+	XVI	+	+	1
	A^-B^+	XVII	+	+	1
	A^-B^-	NA	−	−	5
Clostridium absonum (JCM 1381)	−	NA	−	−	1
Clostridium bifermentans (JCM 1386)	−	NA	−	−	1
Clostridium beijerinckii (JCM 1390)	−	NA	−	−	1
Clostridium histolyticum (JCM 1403)	−	NA	−	−	1
Clostridium novyi (JCM 1406)	−	NA	−	−	1
Clostridium perfringens (JCM 1290)	−	NA	−	−	1
Clostridium perfringens (MRY 05-0166)	−	NA	−	−	1
Clostridium ramosum (JCM 1298)	−	NA	−	−	1
Clostridium septicum (JCM 8144)	−	NA	−	−	1
Clostridium sordellii (JCM 3814)	−	NA	−	−	1
Clostridium sordellii (JCM 11011)	−	NA	−	−	1
Clostridium sporogenes (JCM 1416)	−	NA	−	−	1
Clostridium tertium (JCM 6289)	−	NA	−	−	1

[a] NA, not applicable.
[b] +, positive; −, negative.

Fecal TcdB assay. Stool specimens were tested for TcdB using a Vero cell cytotoxicity assay with a neutralization test with anti-*C. difficile* TcdB serum (TechLab, Blacksburg, VA). The final dilution of stool specimens in each microtiterplate well was 1:100. The cells were examined after both 24 h and 48 h of incubation.

Detection of *tcdB* by LAMP. DNA extraction from cultured isolates for a LAMP assay was performed in the same manner as previously described for PCRs of the toxin genes (12). DNA was directly extracted from stool specimens using the QIAamp DNA stool minikit (QIAGEN, Hiden, Germany) according to the manufacturer's instructions. LAMP was also applied to detection of *tcdB* in DNA which was extracted from overnight cultures of stool specimens with cooked meat medium (Becton Dickinson, Sparks, MD). One swab of stool specimens was inoculated into 5 ml of CMM and incubated at 35°C overnight; 1 ml of inoculated broth was centrifuged at 15,000 × *g* for 2 min, and 500 μl of TES (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) was added to the pellet. The suspension was heated at 95°C for 15 min and centrifuged at 15,000 × *g* for 2 min, and the resultant supernatant was used as the template DNA for the LAMP assay.

The six primers used for the LAMP were derived from *tcdB* (4) (Fig. 1). The outer primers were HK101-F3 (5′-GTATCAACTGCATTAGATGAAAC-3′) and HK101-B3 (5′-CCAAAGATGAAGTAATGATTGC-3′); the inner primers were primer HK101-FIP, consisting of HK101-F1c and HK101-F2 (5′-CTGCACCTA AACTTACACCATCTATCTTCCTACATTATCTGAAGGATT-3′), and primer HK101-BIP, consisting of HK101-B1c and HK101-B2 (5′-GAGCTAAGTGAA ACGAGTGACCCGCTGTTGTTAAATTTACTGCC-3′). The loop primers were primers HK101-FL (5′-AATAGTTGCAATTATAGG-3′) and HK101-BL (5′-AGACAAGAAATAGAAGCTAAGATAGG-3′) (Fig. 1).

The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. We added 2 μl of DNA template to a total volume of 25 μl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers, and 20 pM of each of the loop primers. Amplification was performed at 62°C for 60 min, followed by incubation at 80°C for 2 min to terminate the reaction. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical Co., Ltd., Tokyo, Japan). The turbidity was calculated based on the ratio of light intensity (intensity of light received by the photodiode/emitted light intensity). A ratio of 0.1 was defined as positive for the LAMP assay (20).

Detection of *tcdB* and *tcdA* by nested PCRs in stool specimens. DNA extracted from stool specimens for the LAMP assay was also used as the template for a nested PCR. The primers used for the nested PCR detecting *tcdB* were NK201 (5′-TTTAGATACTACACACGAAG-3′) and NK202 (5′-GCCATTATACCT ATCTTAGC-3′) for the outer primer set and NK104 and NK105 (12) for the inner primer set (Fig. 1), which were derived from *tcdB* (4). A nested PCR

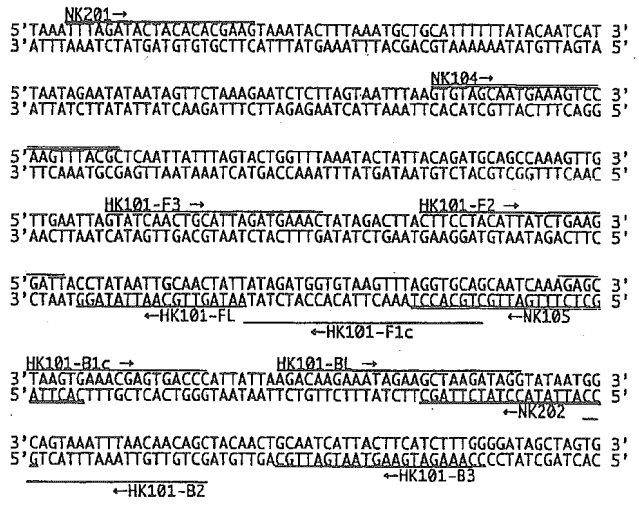

FIG. 1. Oligonucleotide primers used for amplification of *tcdB*. Single-underlined and double-underlined letters indicate the sequences of primers for LAMP and for nested PCR, respectively.

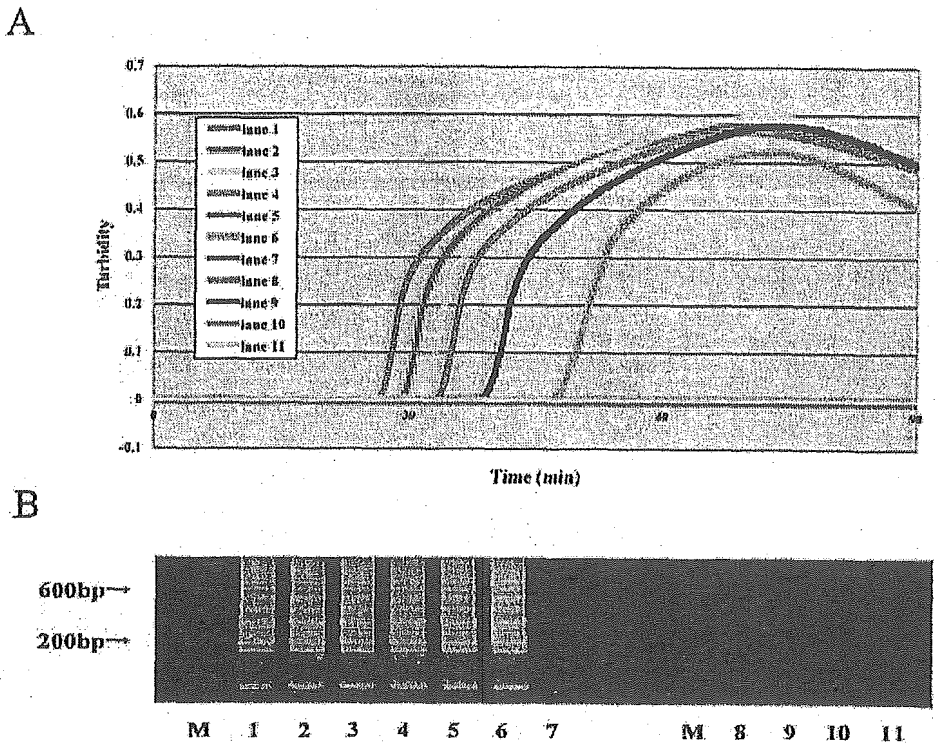

FIG. 2. Real-time detection of turbidity (A) and 5% polyacrylamide gel electrophoresis (B) of amplification products by LAMP. The template extracted from strain VPI 10463 in 10-fold serial dilutions from 50 ng to 50 ag per reaction tube (lanes 1 to 10) was added. Lane 11, negative control; lanes M, 100-bp ladder as a molecular size marker.

detecting the nonrepeating sequences of *tcdA* was performed with primer sets HK5 and HK6 for the outer primer and NK3 and NK2 for the inner primer (13). The nested PCR assay on DNA extracted from stool specimens was performed as described previously (13).

RESULTS

Sensitivity and specificity of LAMP. A total of 40 clinical isolates of *C. difficile* were examined for detection of *tcdB* by LAMP (Table 1). *tcdB* was detected in all 28 A^+B^+ and seven A^-B^+ isolates representing nine and three different toxinotypes, respectively (23). All five A^-B^- isolates examined were LAMP negative for *tcdB*. The test results by LAMP completely agreed with those by PCR detecting *tcdB* with primer set NK104 and NK105. The LAMP was performed in a 90-min reaction to confirm the specificity in 13 strains of 11 *Clostridium* species other than *C. difficile*, with negative results (Table 1).

DNA was extracted from strain VPI 10463 (A^+B^+), and 10-fold serial dilutions from 50 ng to 50 ag of DNA were added to each reaction tube for the LAMP and the nested PCR. Amplification by LAMP was obtained in reaction tubes containing from 50 ng to 0.5 pg of DNA template within 60 min (Fig. 2). Electrophoretic analysis (Fig. 2B) of the final products showed stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops (20, 21). On the basis of the results, the LAMP assay was performed in a 60-min reaction for the following tests. The same serial dilutions of DNA were applied to the nested PCR; the single PCR by primer set NK201 and NK202 was 10-fold less sensitive and the nested PCR was 100-fold more sensitive than the LAMP method (data not shown).

Direct detection of *tcdB* by LAMP in stool specimens. The results of detection of *tcdB* by the LAMP assay in DNA extracted directly from stool specimens compared with those of other tests are shown in Table 2. Of 74 stool specimens examined, 68 were available for detection of TcdB by cell culture assay; 32 were positive for the detection of fecal TcdB and 35 were negative, and the test result was nonspecific in the remaining 1. Amplification of *tcdB* by LAMP was obtained in all stool specimens that were positive for fecal TcdB. All 74 stool specimens were cultured for *C. difficile*; 40 were culture positive and 34 were negative. Of 40 isolates recovered from those specimens, 38 were A^+B^+, one was A^-B^+, and the remaining one was A^-B^-. *tcdB* was detected by LAMP on DNA extracted from 38 of 39 stool specimens from which an A^+B^+ or A^-B^+ *C. difficile* strain was recovered. Direct detection of *tcdB* by LAMP was positive in 10 stool specimens that were negative for *C. difficile* by culture.

The results of direct detection of *tcdB* by LAMP were compared with those by a nested PCR assay. All of the specimens that were positive by LAMP were also positive by nested PCR. The nested PCR detecting *tcdB* generated a PCR product on DNA extracted from 16 stool specimens that were culture negative, of which 10 specimens were positive for LAMP and 6 were negative. A nested PCR detecting *tcdA* was performed on 15 of these 16 specimens, all of which were positive. No specimens were negative for the nested PCR but positive for other tests.

TABLE 2. Results of LAMP assay detecting *tcdB* in DNA extracted from stool specimens and overnight culture in CMM and comparison to other tests

TcdB detection[a] in stool specimen by cell culture	*C. difficile* culture		Direct detection of *tcdB* in stool specimen by:		Detection of *tcdB* from overnight culture in CMM by LAMP	No. of stool specimens
	TcdB detection	Tcd type of isolate	Nested PCR	LAMP		
+	+	A^+B^+	+	+	+	16
+	+	A^+B^+	+	+	−	2
+	+	A^+B^+	+	+	ND[c]	13
Invalid	+	A^+B^+	+	+	+	1
+	+	A^-B^+	+	+	+	1
−	+	A^+B^+	+	+	+	3
−	+	A^+B^+	+	−	+	1
−	+	A^-B^-	−	−	−	1
−	−	NA[b]	+	+	+	2
−	−	NA	+	+	−	5
−	−	NA	+	+	ND	1
−	−	NA	+	−	+	2
−	−	NA	+	−	−	3
−	−	NA	+	−	ND	1
−	−	NA	−	−	−	10
−	−	NA	−	−	ND	6
ND	+	A^+B^+	+	+	ND	2
ND	−	NA	+	+	ND	2
ND	−	NA	−	−	ND	2

[a] +, positive; −, negative. Invalid, test result invalid because of atypical cytotoxic effect.
[b] NA, not applicable.
[c] ND, not done.

Detection of *tcdB* by LAMP from overnight cultures of stool specimens in CMM. A total of 47 stool specimens were available for evaluation of the LAMP assay detecting *tcdB* on DNA extracted from overnight cultures of stool specimens in CMM (Table 2). Of the 47 stool specimens examined, 24 were positive for fecal TcdB and/or for culture of *C. difficile* of A^+B^+ or A^-B^+ on CCMA, of which 22 specimens were positive by LAMP for overnight cultures in CMM and 2 were negative. The two specimens that were LAMP negative in overnight cultures were positive for direct detection of *tcdB* in stool specimens by both LAMP and nested PCR. Amplification by LAMP was obtained on DNA extracted from overnight cultures of four specimens that were negative for both fecal TcdB and *C. difficile* culture on CCMA. The four CMM tubes in which *tcdB* was detected by LAMP were inoculated, and A^+B^+ *C. difficile* could be recovered from all four specimens. Of those four specimens, two were positive for direct detection of *tcdB* from stool specimens by both LAMP and nested PCR, and two were positive only by nested PCR.

DISCUSSION

LAMP is a novel nucleic acid amplification method using DNA polymerase with strand displacement activity and six primers that recognize eight regions on the target nucleic acid, leading to extremely high specificity (20, 21). In the present study, we successfully identified TcdB-positive (A^+B^+ and A^-B^+) *C. difficile* strains with various toxinotypes by LAMP. Recent reports (1, 11, 16, 17, 18) have demonstrated the clinical significance of A^-B^+ strains. Most of the A^-B^+ strains are known to belong to toxinotype VIII (23, 24) and produce a variant toxin B ($TcdB_{1470}$) (12, 25). The primers used for the LAMP assay here could detect $tcdB_{1470}$ as well as other variant types of *tcdB* produced by toxinotypes III and IV (24) that could not be detected by real-time PCR (5). No amplification was observed from TcdB-negative (A^-B^-) *C. difficile* strains or 13 strains of other *Clostridium* species, including two *Clostridium sordellii* strains, which produce the lethal toxin (TcsL), indicating the specificity of the LAMP. Although the LAMP assay used here cannot distinguish A^-B^+ strains from A^+B^+ strains, identification of TcdB-positive *C. difficile* should be important for clinical diagnosis.

The LAMP detecting *tcdB* in DNA extracted directly from stool specimens proved to be a reliable assay when the test results were compared with those for detection of fecal TcdB and *C. difficile* culture. Furthermore, amplification was obtained by direct LAMP in 10 specimens that were negative for both fecal TcdB and culture, indicating the LAMP is more sensitive than culture for some specimens. Positive results in nested PCRs for not only *tcdB* but also *tcdA* in LAMP-positive but culture-negative specimens indicate the presence of PaLoc sequences in specimens and the specificity of the LAMP assay. Two of 10 patients from whom the LAMP-positive but culture-negative specimens were obtained were on vancomycin therapy when the specimens were tested, which should be one of the reasons for the lack of *C. difficile* growth. Although the nested PCR proved its high sensitivity, it is not of practical use in clinical laboratories because the procedure is time-consuming and tedious and due concern must be paid to contamination of PCR products.

Although the QIAamp DNA stool minikit is useful for extraction of DNA from stool specimens (2, 9, 13), it is not always practical for clinical laboratories. The LAMP method was applied to the detection of *tcdB* in DNA extracted by a simple

and quick boiling method from stool specimens which were cultured overnight in CMM, and a positive LAMP reaction was successfully obtained for 22 of 24 culture-positive specimens. Two specimens were culture positive but LAMP negative on DNA extracted from CMM culture. This discrepancy might be explained by the existence of amplification inhibitors in samples, because DNA was extracted without any steps for removing inhibitory substances. The heterogeneity of stool specimens also might cause the discrepancy when the specimens contain a low number of *C. difficile* or contain mucus.

Interestingly, four stool specimens that were negative for culture on CCMA were LAMP positive in overnight-cultured CMM. Recovery of *C. difficile* from of these four specimens in CMM indicated that the results of the LAMP with CMM cultures were not false-positives. This simple method using CMM requires neither tedious steps for DNA extraction from stool specimens nor anaerobic incubation equipment, such as an anaerobic chamber, jar, or pouch, making it possible to perform the test at clinical laboratories without special apparatus. In addition, the hands-on time of the procedure is very short, even though the test results are provided on the day after specimen collection.

The LAMP assay is a novel method to amplify DNA under isothermal conditions (20, 21) and has been applied to the identification or detection of some bacteria with high sensitivity and specificity (8, 10). The method is more rapid and easier to perform than a PCR assay and does not require any special equipment, such as a thermal cycler or electrophoresis system. The turbidimeter used in the present study is not needed when the fluorescent detection reagent (Eiken Chemical Co., Ltd., Tokyo, Japan) and UV lamp are available.

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NOTE

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Emergence of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* in tuberculosis wards

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Abstract To assess whether the occurrence of rifampicin (RFP) resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) is related to treatment of tuberculosis, we determined the RFP susceptibility of MRSA isolates obtained from tuberculosis patients and screened for mutation(s) in the *rpoB* gene of these isolates. The MICs of RFP for 84 MRSA isolates obtained from two hospitals in Japan were determined. DNA was sequenced in the region 1318–1602 nucleotides (nt) of the *rpoB* gene, which includes RFP resistance-determining clusters I (1384–1464 nt, 462–488 amino acids). The majority of MRSA isolates from tuberculosis wards, i.e., 48 of 51 (94%) [33 of 34 in a Tokyo hospital (97%) and 15 of 17 in a Chubu hospital (88%)], were resistant to RFP. Meanwhile, no isolates of 33 from the other wards were resistant to RFP. All RFP-resistant MRSA isolates had a mutation(s), including novel mutation(s) such as Val453→Phe, Asp471→Asn, and Ile527→Leu, in *rpoB*. An emergence of RFP-resistant MRSAs in tuberculosis wards in Japan was strongly suggested.

Key words Rifampicin · Drug resistance · MRSA · *rpoB* · Tuberculosis

Rifampicin (RFP) is one of the first-line antituberculous agents and also a potent antimicrobial agent against methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] RFP acts by interacting in a specific manner with the β-subunit of the bacterial RNA polymerase encoded by the *rpoB* gene.[3] In MRSA infections, RFP is often used in combination with antibiotics with lower penetrability, such as vancomycin.[4,5] The combination therapy with RFP revealed strong activity and good tissue penetration that is required to reach deep-seated infections effectively.[4,5] In such a situation, there is a high risk of emergence of RFP-resistant MRSA. Most RFP-resistant MRSA organisms and other bacteria are known to have a mutation(s) in the particular regions, clusters I and II in the *rpoB* gene encoding the RNA polymerase β-subunit.[4–7]

In the present study, we examined RFP susceptibility of MRSA isolates obtained from inpatients with tuberculosis and screened for mutations in the *rpoB* gene of these isolates. A total of 84 MRSA isolates obtained from hospitals in Tokyo[8–12] and Chubu district[13] were analyzed. *S. aureus* ATCC29213 and ATCC700699 strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Of these isolates, 51 were obtained from tuberculosis wards in both hospitals (34 from a hospital in Tokyo during an MRSA outkreak in 2001[12] and MRSA surveillance studies done before and after the outbreak in 2000–2003[8–11] and 17 from a hospital in Chubu during an MRSA outbreak[13]), and 33 other isolates were from other wards in a Tokyo hospital.[8] All MRSA isolates were analyzed by pulsed-field gel electrophoresis (PFGE) as described previously.[8–13] Differences between tuberculosis wards and the other wards in the isolation numbers of MRSA were analyzed by Fisher's exact probability test. A P value <0.05 was considered statistically significant.

The minimum inhibitory concentration (MIC) of RFP was determined by an E-test (AB BIODISK, Dalvagen, Sweden), and the result was interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards.[14] The staphylococcal breakpoint for resistance to RFP is defined as ≥4 μg/ml (susceptible is defined as ≤1 μg/ml).[14]

The distribution of RFP MICs for the MRSA isolates obtained from tuberculosis and other wards is shown in Fig. 1. The MICs of RFP ranged from ≤0.002 to ≥256 μg/ml.

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Among the 84 MRSA isolates, 48 were resistant to RFP with MIC ≥48 μg/ml. The other isolates were susceptible to RFP with MIC ≤0.015 μg/ml. The majority of MRSA isolates from tuberculosis wards, i.e., 48 of 51 (94%) [33 of 34 in a Tokyo hospital (97%) and 15 of 17 in a Chubu hospital (88%)], were resistant to RFP (Fig. 1, Table 1). Meanwhile, 0 of 33 isolates from the other wards were resistant to RFP ($\chi^2 = 72.47$, $P < 0.001$) (see Table 1).

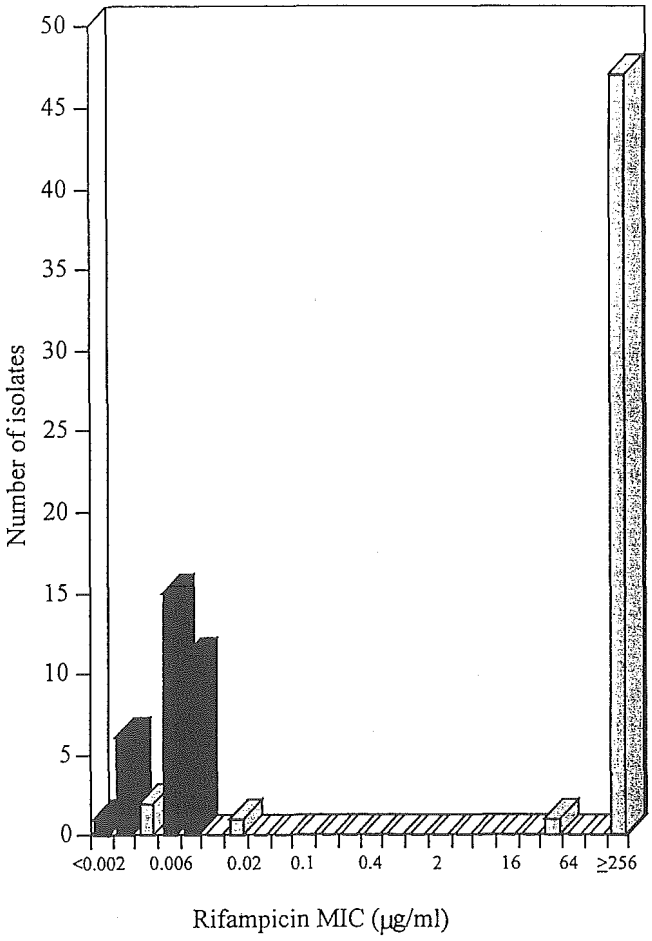

Fig. 1. Distribution of rifampicin minimum inhibitory concentrations (*MICs*) for 84 methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in Tokyo and Chubu hospitals. *Gray bars* represent MRSA isolates obtained from tuberculosis wards; *black bars* represent MRSA isolates from other wards

The DNA sequence of the region of 1318–1602 at nucleotide positions (nt) of *rpoB*, corresponding to codons 440–534 (amino acid number, aa number), which includes the RFP resistance-determining cluster I (1384–1464 nt, 462–488 aa)[4] and cluster II (1543–1590 nt, 515–530 aa)[4] of *S. aureus* were amplified by polymerase chain reaction (PCR) with the primers rpoB-F (5′-CCG TCG TTT ACG TTC TGT AGG-3′) and rpoB-R (5′-AAA GCC GAA TTC ATT TAC ACG-3′). PCR products were sequenced with the same primers by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA). Of 84 isolates analyzed, 32 had one mutation and 16 had two mutations in clusters I and II of *rpoB* (Table 2). A total of 64 mutations were identified, and all mutations resulted in amino acid substitution. Of them, 60 mutations were located in cluster I: 19 were Ala 477→Asp, 14 were Ser 486→Leu, 12 were His 481→Asp, 12 were Ala 473→Thr, 1 was Ser 464→Pro, 1 was Gln 468→Leu, and 1 was Asp 471→Asn. Three mutations were located in cluster II; all three were Ile 527→Leu. One was found in the region upstream from cluster I, i.e., Val453→Phe. All mutations except for the three mutations, Asp471→Asn, Ile527→Leu, and Val453→Phe, were already reported to be related to RFP resistance in *S. aureus*.[4–7] Type 3 isolates were resistant to RFP and had a single mutation of Asp471→Asn, indicating that the *rpoB* mutation was associated with RFP resistance. The mutations at 527 aa, Ile527→Phe or Ile527→Met, were known to be related to RFP resistance.[2] However, whether the mutation Ile527→Leu at the same position was associated with RFP resistance is unclear, because additional mutations known to be related to RFP resistance were present (see type 4 and 5 isolates, Table 2). The association of Val453→Phe with RFP resistance is also unclear because there was an additional mutation associated with RFP resistance (see type 11 isolates, Table 2). Nevertheless, three novel mutations of Asp471→Asn, Ile527→Leu, and Val453→Phe were identified in *S. aureus*.

Based on RFP susceptibility testing, PFGE genotyping, and DNA sequencing of *rpoB*, the MRSA isolates from tuberculosis wards were classified into 23 types (see Table 2). Among 84 isolates, 12 isolates from a Chubu district hospital (type 7) were resistant to RFP (MIC, >256 μg/ml), showed PFGE pattern A2(M1), and had a mutation of Ala 477→Asp; 11 isolates from a Tokyo hospital (type 12) were resistant to RFP (MIC, >256 μg/ml), showed PFGE pattern

Table 1. Frequency of rifampicin-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in tuberculosis wards

Rifampicin susceptibility	No. (%) of isolates			
	Tuberculosis wards			Other wards
	Tokyo[a] ($n = 34$)	Chubu district[b] ($n = 17$)	Total ($n = 51$)	T ($n = 33$)
Resistant	33 (97%)	15 (88%)	48 (94%)	0 (0%)
Susceptible	1 (3%)	2 (12%)	3 (6%)	33 (100%)

[a] Tokyo hospital
[b] Chubu district hospital

Table 2. Resistance to rifampicin and mutations in the *rpoB* gene of *S. aureus* in tuberculosis wards

Hospital[a]	Specimen or reference strain	No. of isolates	Rifampicin MIC (μg/ml)	PFGE genotype[b]	*rpoB* gene		Type no. assigned
					Nucleotide changes[c]	Amino acid changes[d]	
T	Sputum	1	48	A14	TCT→CCT	Ser464→Pro	1
T	Sputum	1	>256	A2(M1)	CAA→CTA	Gln468→Leu	2
T	Sputum	1	>256	F6	GAC→AAC	Asp471→Asn[e]	3
T	Sputum	1	>256	F2	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	4
T	Sputum	2	>256	F4	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	5
C	Gastric juices	1	>256	M2(A18)	GCT→GAT	Ala477→Asp	6
C	Sputum	12	>256	A2(M1)	GCT→GAT	Ala477→Asp	7
C	Sputum	1	>256	AO	GCT→GAT	Ala477→Asp	8
C	Sputum	1	>256	M7	GCT→GAT	Ala477→Asp	9
T	Sputum	1	>256	G2	GCT→GAT	Ala477→Asp	10
T	Sputum	1	>256	J1	GTT→TTT, TCA→TTA	Val453→Phe[e], Ser486→Leu	11
T	Arterial blood	1	>256	J1	TCA→TTA	Ser486→Leu	12
	Sputum	9					
	Nasal cavity	1					
T	Nasal cavity	1	>256	J2	TCA→TTA	Ser486→Leu	13
T	Sputum	1	>256	J4	TCA→TTA	Ser486→Leu	14
T	Thorax drain	1	>256	R1	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	15
	Sputum	1					
T	Sputum	3	>256	J7(R2)	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	16
	Arterial blood	1					
	Urine	1					
T	Sputum	1	>256	J8	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	17
T	Sputum	1	>256	A1	CAT→TAT	His481→Asp	18
T	Sputum	1	>256	A2(M1)	CAT→TAT	His481→Asp	19
T	Urine	1	>256	S	CAT→GAT	His481→Asp	20
T	Sputum	1	>256	A22	CAT→TAT	His481→Asp	21
T	Sputum	1	0.015	AU1	No change	No change	22
C	Sputum	2	0.005	A3	No change	No change	23
	ATCC29213		0.005	–	No change	No change	
	N315		0.004	–	–	–	

MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis
[a]T, Tokyo hospital; C, Chubu district hospital
[b]Data from references 8–13
[c]Base changes are underlined
[d]The numbering of the amino acids is based on that of *S. aureus* N315 (GenBank accession no. NC-002745)
[e]Novel mutation

J1, and had mutation Ser486→Leu; and 5 isolates from Tokyo (type 16) were resistant to RFP (MIC, >256μg/ml), showed PFGE pattern J7(R2), and had two mutations of Ala 473→Thr and His 481→Asp, indicating that there was clonal expansion of these RFP-resistant MRSA strains in tuberculosis wards in both hospitals. Sixteen isolates of types 1–4, 6, 8–11, 13, 14, and 17–21 were resistant to PFP, but showed different genotypes (PFGE patterns and *rpoB* mutations), indicating that individual strains of RFP-resistant MRSA existed in tuberculosis patients. Collectively, these results suggest that there were two types of transmission mode of MRSA isolates: some were transmitted within tuberculosis wards and the others were brought from outside the wards.

In conclusion, MRSAs obtained from tuberculosis wards in two hospitals in Japan had resistance to RFP and mutation(s) in the particular regions of *rpoB*. It is difficult to conclude that RFP-resistant MRSA isolates were emerging in the wards during RFP therapy. Nevertheless, the present results strongly suggest an emergence of such MRSAs in tuberculosis wards in Japan. It is necessary to monitor PFP resistance in both tuberculosis and other wards.

The DNA sequences of part of the *rpoB* of MRSA reported here were registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB195713, AB195714, and AB195715.

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病院感染対策の基本

組織としての対応を理解する

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5

組織としての対応を理解する

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感染症は微生物の身体への侵入が発症原因であるが，宿主の防御機構（解剖学的構造および免疫能・感受性）の他に宿主の生活する環境（施設の構造および運営システム）が重要な因子となっている．そのため環境感染の観点から，病院感染を管理する必要がある．すなわち，施設の空調などの構造・設備の改善や運営方針の決定など，組織として病院感染に対応しなければならない．また，病院感染の防止は，感染発症や感染伝播に対する個人個人の知識と医療技術が基礎とはなるが，一部の職員の油断が二次感染拡大に直接つながることからも，組織としての対応が重要である．種々の規程や管理を決める感染対策委員会 Infection Control Committee（ICC）と，実働部隊である感染対策チーム Infection Control Team（ICT）が大きな役割を果たす．

ICCとICT，リンクナースの存在意義と役割

病院感染は，「医療事故の1つ」であるとの認識により，患者および職員の安全管理の観点から病院長の諮問機関である各種委員会の1つではなく，病院長直属の組織とすることが望ましい．すなわち，ICCの委員長は病院長もしくは看護部長など管理職が担当し，その委員会での議決がそのまま直接組織としての決定事項となり，即座に実行へと移される体制である．そのために，委員会のメンバーには，感染症や微生物学の専門家以外に，予算執行の責任者である会計課長や種々の条例解釈や規程の担当である庶務課長などの事務職員，外来，手術室，検査室，薬剤部などの責任者が入っていることが好ましい．したがって，ICCの委員は個人名による指名ではなく，役職で決めることが重要である．

現場での指導や相談対応はICTが行うことになる．現場でのマニュアル，手順書の活用，問題が発生した場合の相談と対処方法など，即座に対応しなければならない場合もあるため，少なくとも数人の専任は必要であろう．また，マニュアルの定期的見直しや改定，抗菌薬の使用指針，分離菌の種類と抗菌薬感受性の推移の情報発信，ターゲットサーベイランス実施と評価，職員・出入り業者への教育研修など，ICTの役割は多種にわたり，また重要である．ICTの提案はICCで承認されなければならない．

病院感染は，外来の待合室等でも二次感染という形で発生することもあるが，通常は入院後48時間以降に発症した感染で，感染症の潜伏期に入院したものを除くということになっている．したがって，病院感染の舞台は病棟ということになるが，一番患者に接し観察しているのは医師でなく看護師である．そのために最前線の感染管理はリンクナースがキーパーソンとなる．リンクナースとICTの連携とそれらの役割を，職員が充分に理解してはじめて実効性を発揮する．

運営方針

職員の健康管理は，感染の伝播の観点とともに感染源の観点からも，病院感染対策の第一歩である．職員採用時の健康診断では既往歴や予防接種歴の確認が重要で，特に結核，麻疹，水痘など空気感染する疾患，B型肝炎など事故により感染する疾患に関しては本人の申請だけでなく胸部X線写真や血清抗体価などで客観的に確認し，結核予防法や労働基準法などの条例に規程のない対応

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