the pre-vaccination period has been estimated to be $R_0 = 10$–12 (Anderson and May, 1991). More recent estimates have been $R_0 = 10$–15 in countries with poor sanitation and hygiene, and R_0 less than 10 in countries with good sanitation and hygiene (Fine and Carneiro, 1999). If we assume $\gamma = 0.18$, this gives estimates of $\beta = 1.8$–2.7/week in developing countries. Much higher R_0's of more than 20 have been reported by studies of poliomyelitis outbreaks over the past 20 years (Patriarca et al., 1997). Because of this large variance in the estimated β, we varied the value rather widely, from 2 to 6, to evaluate eradication probability.

Mutation rate μ from the attenuated to the virulent virus

Oral polio vaccine produced from Sabin 1 to 3 strains is a highly attenuated vaccine. It is known that virulent mutants appear after replication in the human gut after OPV given. Such virulent strains have caused outbreaks in populations with low OPV coverage in Haiti, the Dominican Republic, and Egypt, accumulating mutations through human to human transmission (Centers for Disease Control and Prevention, 2000, 2001). Several nucleotide mutations responsible for attenuation have ever been reported (Plotkin et al., 2008). Of them, the critical and unstable attenuating mutations in 5′-UTR (A480G in Sabin 1, G481A in Sabin 2, and C472U in Sabin 3) appear initially during viral replication. Dunn et al. (1990) reported that at least one viral serotype excreted from a susceptible individual immunized by OPV had mutated completely in 5′-UTR within 28 days. The average contents of revertants (virulent forms) from OPV recipients were 28–40% in type 1, 97% in type 2, and 67% in type 3 at 3 weeks after the most recent dose (Laassri et al., 2006). Similar estimates were reported by Minor et al. (2005) and Martinez et al. (2004). Thus, the mutation rate from attenuated to virulent viruses appeared to be high, in the order no smaller than $\mu = 0.1$/week.

RESULTS

Before proceeding to specific parameter dependences, it should be noted that the time at which the fraction of susceptible hosts exceeds the threshold for epidemics is crucial in understanding the problem. The number of virulent virus infected hosts increases if the fraction of susceptible hosts is larger than the threshold $x_c = (u+\gamma)/\beta$, which is the reciprocal of the basic reproductive rate $R_0 = \beta/(u+\gamma)$, and decreases when x is smaller than x_c. During the initial period, when the fraction of OPV-vaccinated individuals is large, the fraction of susceptibles is less than the threshold x_c, so that the risk of an outbreak is negligible, even though considerable numbers of virulent mutants are being generated at each time step. The number of virus carriers decreases during the period from the cessation of OPV to time t_c at which the susceptible density exceeds the threshold x_c. If the number of carriers becomes zero around t_c, polio will be globally eradicated. However, if virus survives this "endangered" period around t_c, the infected density increases again and a future outbreak becomes certain. The following formula (derived in Appendix) provides an approximate time t_c and minimum infected fraction w_c as a function of epidemiological parameters:

$$t_c \approx Lp/R_0, \quad (R_0 >> 1), \tag{12a}$$

$$Kw_c \approx K\frac{D}{L}exp\left[-\frac{p^2}{2R_0}\frac{L}{D}\right], \quad (R_0 >> 1,\ L >> D), \tag{12b}$$

where $D = 1/\gamma$ is the mean duration of infection, $L = 1/u$ the life expectancy of the host, and $R_0 = \beta/(u+\gamma)$ the basic reproductive ratio. There is a high probability of global eradication if Kw_c is sufficiently smaller than 1; whereas, there is a high-risk of re-emergence if Kw_c is greater than 10. Although assessment of outbreak risk should be based on the probability of global viral extinction as discussed below, the above approximate formula gives insights into the likelihood of re-emergence and parameter dependence on eradication probability. It also gives an accurate estimate of the critical time t_c at which either global eradication occurs or an outbreak starts.

PATHS TO EXTINCTION AND PATHS TO OUTBREAK

Figure 3 shows deterministic changes in fraction x of susceptibles and fraction $w = y + v$ of poliovirus carrying hosts after cessation of live vaccination. The fraction of susceptibles exceeded the epidemiological threshold x_c around time $t = t_c(=150)$ weeks after live vaccination discontinuation. When the fraction of susceptibles exceeds the epidemiological threshold, the fraction of infecteds is at its minimum. The public health objective is to make the number of infecteds zero around time $t = t_c$. **Figure 4** illustrates sample paths for the stochastic process corresponding to the deterministic trajectory in **Figure 3**. In this example, 61 out of 100 independent

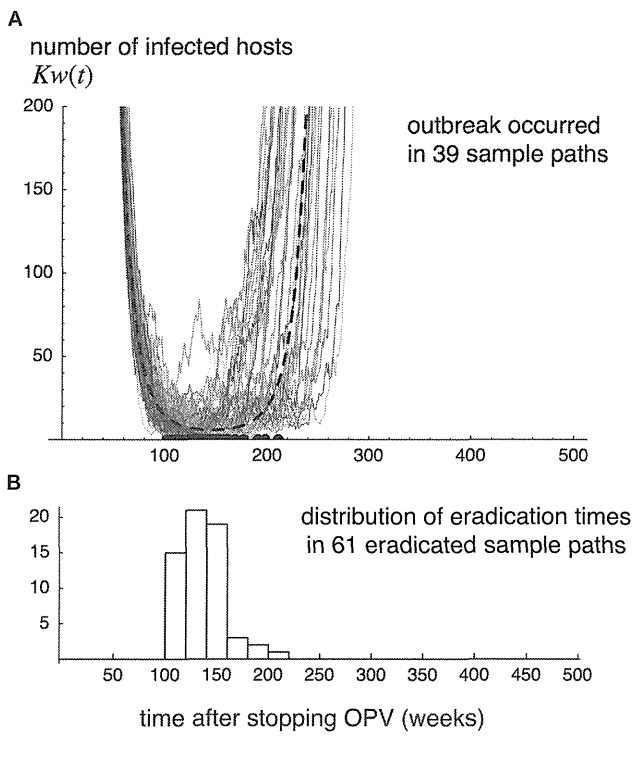

FIGURE 4 | Sample paths for the number of infecteds observed in Monte Carlo simulations. Sample paths for the number of infecteds observed in Monte Carlo simulations of the stochastic process corresponding to dynamics (7). One hundred independent runs are illustrated by thin lines. Thick broken lines indicate the deterministic trajectory **(A)**. The histogram shows the distribution for the times at which viruses went to extinction **(B)**. 38 out of 100 runs never go to extinction, and cause outbreaks. The parameters are the same as in **Figure 3**, and $K = 10^8$.

runs led to the global eradication of poliovirus (i.e., the number of infected hosts hit the absorbing boundary at zero). However, in the remaining runs, poliovirus escaped extinction around $t = t_c$, increased again, leading to an outbreak by a virulent strain. The probability of successful eradication is thus 61% by the parameter set used in **Figure 4**.

PARAMETER DEPENDENCE

Figure 5 illustrates how the probability of the failure of global eradication $P_{\text{fail}} = 1 - P_{\text{ext}}$ depends on each parameter, which we discuss in turn below. We set the following values as "standards," and varied each of the parameters to see its effect. The fraction of immunized newborns before $t = 0$: $p = 0.7$; transmission rate of virulent virus: $\beta_v = 3.7$, that of attenuated virus: either $\beta_a = \beta_v$ or $\beta_a = \beta_v/2$; recovery rate: $\gamma = 0.18$ (in both viruses); mutation rate from attenuated to virulent viruses: $\mu = 0.1$; natural host mortality: $u = 0.00025$ (all measured in units of weeks), and total population: $K = 100$ million. With the chosen values of β, u, and γ, the basic reproductive rate of polioviruses was $R_0 = 20$. In **Figure 5**, lines indicate the eradication probability calculated from Eqs 8–11 for $\beta_a = \beta_v$, the dots indicate the observed eradication probability for 1000 independent runs of the stochastic process corresponding to the deterministic model (7) for $\beta_a = \beta_v$, and the crosses indicate that for $\beta_a = \beta_v/2$. We first discuss the results for $\beta_a = \beta_v$ in Section "The Immunization Fraction p Before Stopping OPV, The Recovery Rate γ, The Transmission Rate β, The Mutation Rate μ From the Attenuated to Virulent Viruses, and The Total Population Size K" below, and discuss the effect of a lower transmission rate of attenuated virus in 3.2.6.

The immunization fraction p before stopping OPV

The effect of fraction p of OPV-immunized newborns before stopping the live vaccination is illustrated in **Figure 5A**. While the probability of failing eradication is low when p is sufficiently high, it rises drastically around $p = 0.7$ when p is decreased. For example, if the immunization fraction is 60% or less before OPV is stopped, future outbreak by virulent poliovirus is almost certain. There are two reasons why a lower p before stopping OPV enhances the risk of future outbreaks: first, it shortens the time for the susceptible host density to reach the epidemiological threshold, and second, it increases the initial infected density w_0, thereby keeping the minimum density from extinction.

The recovery rate γ

The success of global eradication greatly depends on the recovery rate, or its reciprocal, the mean infectious period (**Figure 5B**). The higher the recovery rate, the more rapidly the number of poliovirus carriers decreases after supply by OPV is stopped. It is then possible to make the expected number of infecteds negligibly small when the susceptible fraction exceeds the epidemiological threshold. Conversely, by having a longer infectious period (a lower recovery rate), viruses safely persist over the endangered period around $t = t_c$. In examples shown in **Figure 5B**, infectious periods

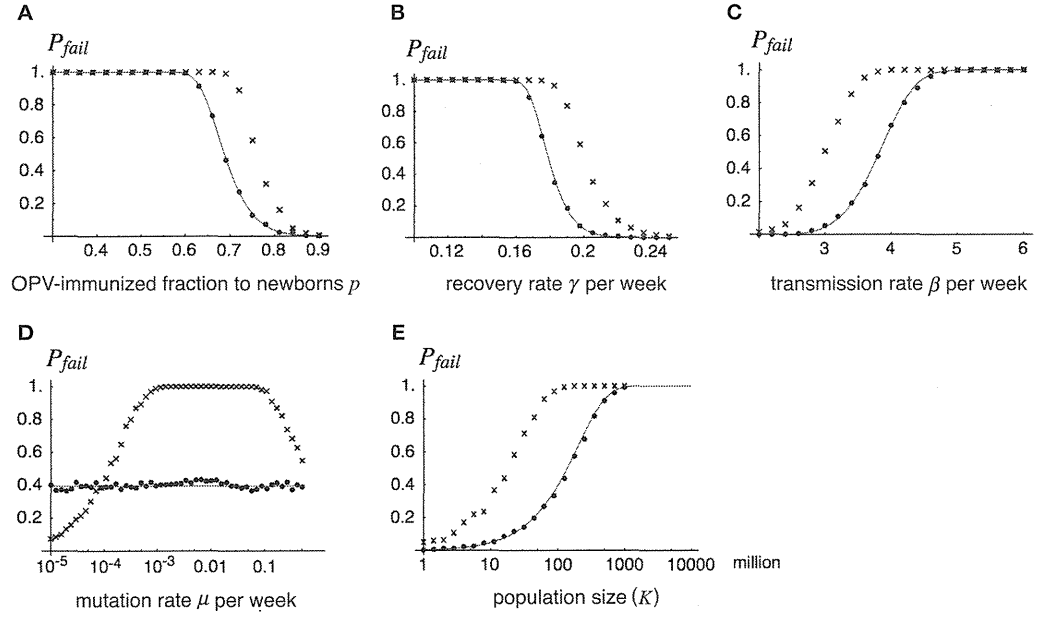

FIGURE 5 | The probability of the failure of global eradication as a function of epidemiological and genetic parameters. Each panel shows how the probability of failing the global eradication $P_{\text{fail}} = 1 - P_{\text{ext}}$ depends on a chosen parameter, where lines show analytical results drawn from P_{ext} defined in Eq. 11, and dots show Monte Carlo simulation results. Except for the varying parameter in each panel, the parameters are fixed as $p = 0.7$, $\beta = 3.7$ ($\beta_v = \beta_a = \beta$ for dots and lines, and $\beta_v = \beta$, $\beta_a = \beta/2$ for cross-hatched), $\gamma = \gamma_v = \gamma_a = 0.18$, $m = 0.1$, $K = 10^8$, and $u = 0.00025$. Varying parameters are: **(A)** fraction p of OPV immunization before its cessation, **(B)** recovery rate γ, **(C)** transmission rate β, **(D)** mutation rate μ, **(E)** total population size K. Lines: the probability of failure obtained from formula (11) in the text (for $\beta_v = \beta_a = \beta$), dots: the proportion of failing eradications in 1000 independent runs of the Monte Carlo simulation for $\beta_v = \beta_a = \beta$, and cross-hatched: that for $\beta_v = \beta$, $\beta_a = \beta/2$.

of 7 weeks or longer are disastrous for eradication. In reality, the infectious period varies between hosts, such that in hosts with innate immunodeficiency the infectious period can be typically longer than 1 year (Hara et al., 1981; Kew et al., 1998). Even a tiny fraction of such hosts significantly increases the risk of virulent virus outbreaks, as we show later.

The transmission rate β

The effect of increasing the transmission rate (**Figure 5C**) is parallel to decreasing the recovery rate described above, and both can be regarded as having the effect of increasing R_0. However, decreasing the recovery rate affects eradication probability more sensitively than increasing the transmission rate, as the former contributes to slowing the decay rate for the number of virus carriers as well as increasing R_0 (see also Eq. 12).

The mutation rate μ *from the attenuated to virulent viruses*

The eradication probability is insensitive to the mutation rate from attenuated to virulent viruses for the case of $\beta_v = \beta_a$ (**Figure 5D**). If viruses persist during the period around $t = t_c$, it does not matter which type survived as eventually the virulent virus increases its relative frequency in the viral population (if $\beta_v = \beta_a$). Quite different results follow when the attenuated virus has a lower transmission rate than the virulent virus (the crosses), where the probability of failing eradication is maximized for an intermediate mutation rate.

The total population size K

This has an obvious dependence on the risk of outbreaks. The larger the population size, the larger the probability that viruses are not lost during the endangered period, and hence, the larger the risk of outbreaks. In the example shown in **Figure 5E**, a population of 10 million individuals has a more than 90% of chance for successful eradication, but communities of 100 and 1000 million have only 50% and less than 5% chances, respectively, using the same epidemiological parameters.

The transmission rate β_a *of attenuated virus smaller than that* β_v *of virulent virus*

In each panel of **Figure 5**, the probability of failing global eradication when the transmission rate β_a of attenuated virus is half of that of virulent virus β_v is plotted as the cross-hatches. In all cases except for the dependence of mutation rate, a lower transmission rate of attenuated viruses *increases* the risk of virulent virus outbreak after the cessation of OPV. This rather counter-intuitive results follow from the fact that silent circulation of attenuated viruses under live vaccination helps increasing the efficiency of immunization, as we have seen in the comparison between the threshold immunization fractions with and without silent circulation [see (2)], and the equilibrium densities for $\beta_a < \beta_v$ (left panels of **Figure 2**) and for $\beta_a = \beta_v$ (right panels). Decreasing the transmission rate of attenuated virus increases the density of susceptibles in the equilibrium population under vaccination, thus shortening the time until the susceptible density hits the epidemiological threshold after the cessation of OPV (compare **Figure 2C** with **Figure 2D**).

TAIL OF INFECTIOUS PERIOD

A constant recovery rate assumed in the previous sections implies that the infectious period is exponentially distributed. One may suspect that an outbreak of vaccine-derived viruses a few years after the cessation of OPV might be the artifact caused by this long tail in the infectious period. We found, however, that the long tail in the infectious period is not necessary for this to happen – it is the silent circulation of avirulent polio viruses in the population, commonly observed in nature and occurring in our model as well, that is responsible for the outbreak that occurs long after the cessation of OPV. To show this, we conducted numerical simulations in which we assume that the host recovers exactly 4 weeks after the infection, i.e., the distribution of infectious period has no tail at all. The infected hosts nevertheless persist in the population far longer than 4 weeks (the infectious period of an individual) after stopping OPV, which allows the outbreak of vaccine-derived strain to occur a few years after the cessation (**Figure 6**).

MARGINAL RISK OF OUTBREAK

Figure 7 illustrates change over time in the marginal risk of viruses found at time t. Marginal risk is defined as $1 - q(t)$ – the probability that an infected host present at time t harbors viruses whose progeny will cause a future outbreak. Marginal risk is negligibly small just after $t = 0$, and rapidly increases with t near $t = t_c$. In the parameters used in **Figure 7**, the rate of increase in probability is the highest around $t = 150$ when the susceptible host density exceeds the threshold (see **Figure 3**). However, the marginal risk of viruses before this point is by no means negligible as there is notable probability that progenies of viruses found during $t = 100$–150 would later cause an outbreak.

EFFECT OF A HIGH-RISK GROUP

We here examine the case where a small fraction r of hosts has a recovery rate, γ', much lower than γ for other hosts. In the simulation shown in **Figure 8**, the recovery rate of most individuals was $\gamma = 0.2$. Using this value, successful eradication is certain (other parameters: transmission rate, $\beta = 2.5$; natural mortality, $u = 0.00025$; immunization fraction before stopping OPV, $p = 0.7$; total population, $K = 100$ million). When we assume only 0.01% of newborns have a 10-times longer infectious period than other members, i.e., $\gamma' = 0.1\gamma$, due to innate (World Health Organization, 1989; Fine and Carneiro, 1999), or acquired immunodeficiency, the probability of failure in global eradication rises to 79% (**Figure 8**). Thus even a tiny fraction of high-risk group drastically makes the global eradication difficult.

EFFECTIVENESS OF IPV

What if extensive IPV immunization follows the cessation of OPV? We assume in this case that all newborns are immunized by inactive vaccine before eventual eradication. The probability of global eradication is then evaluated in the light of the results obtained so far by replacing the transmission rates and recovery rates with values for previously IPV-immunized hosts instead of the values for susceptible hosts. IPV cannot prevent infection by either attenuated or virulent viruses, although it can reduce disease severity, and fewer viruses are excreted from IPV-immunized hosts than from

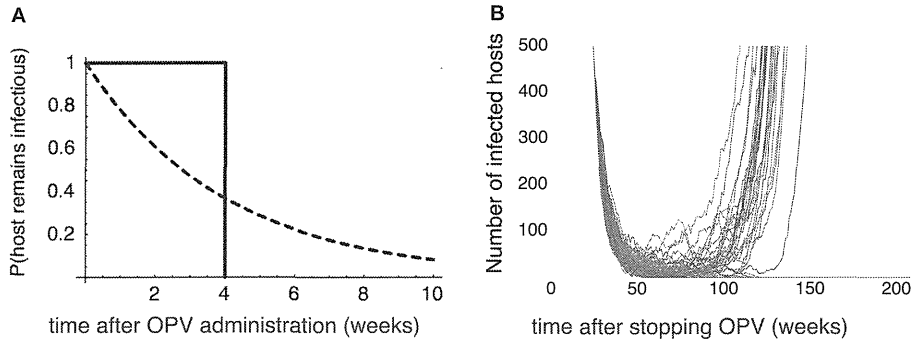

FIGURE 6 | The effect of tail in the infectious period. (A) The probability that the host remains infectious after it is infected at time 0. Dotted curve: the exponential distribution assumed in the previous sections with a constant recovery rate $\gamma = 0.25$/week. Solid curve: the truncated distribution in which all the hosts recovers exactly 4 weeks after the infection. **(B)** The Monte Carlo simulation results assuming the truncated distribution of the infectious period. The time change in the number of virus infected hosts since OPV is stopped. Twenty-six out of 100 runs never go to extinction, and cause outbreaks. The emergence of virulent virus occurs after 50–60 weeks after the secession of OPV. The parameters are $\beta_a = 2.5$, $\beta_v = 5$, $u = 0.00025$, $p = 0.6$, $\mu = 0.1$, and $K = 10^8$. The "mean" infectious period is 4 weeks.

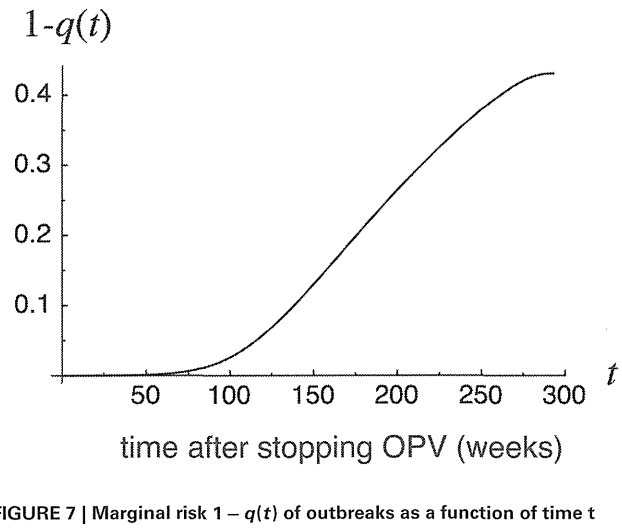

FIGURE 7 | Marginal risk $1 - q(t)$ of outbreaks as a function of time t since OPV cessation. The marginal risk $1 - q(t)$ is defined as the probability that an infected host present at time t harbors viruses whose progeny will cause outbreaks in the future. $p = 0.7$, $\beta = 3.7$, $\gamma = 0.18$, $u = 0.00025$, $K = 10^8$.

unvaccinated hosts (Henry et al., 1966). IPV vaccination would therefore reduce the transmission rate and increase the global eradication probability (see **Figure 5C**). Also, IPV immunization reduces the infectious period, again increasing the probability of successful eradication (**Figure 5B**). However, these considerations assume that *all* hosts are IPV-immunized after the cessation of OPV. The actual amount of risk reduction by IPV depends on coverage, vaccine efficiency, and host heterogeneity in the excretion period.

DISCUSSION

The PAHO and WPRO (Regional Office for the Western Pacific) declared the eradication of poliomyelitis in 1994 and 2000, respectively. Nevertheless, an outbreak of poliomyelitis caused by a type 1 vaccine-derived strain was reported in Haiti and the Dominican Republic in 2000 (Centers for Disease Control and Prevention, 2000), and an outbreak by a type 2 vaccine-derived strain has been reported in Egypt (Centers for Disease Control and Prevention, 2001), in Nigeria (Wassilak et al., 2011). It is assumed that both cases were due to the low rate of vaccine coverage. Although OPV or IPV immunization have been effective in controlling the transmission of wild-type strains, cases of re-emergence by wild-type strains have been reported in several countries (Patriarca et al., 1997) in which inadequate vaccine potency or a high rate of unimmunized individuals led to low herd immunity in the population.

According to a review by Patriarca et al. (1991) rates of seroconversion by OPV approached 100% for each serotype in industrialized countries, but were ~70% for types 1 and 3 in developing countries. Many studies have demonstrated that interference by enteroviruses in human gut and other factors in OPV administration affect the seroconversion rate (Triki et al., 1997). Thus, even if OPV coverage is as high as 90%, the immunized fraction p in our model becomes 62%, under the 70% seroconversion rate observed in developing countries. This should invoke serious concern if we recall that the reduction in immunization fraction p before cessation of OPV drastically increases the risk of outbreak, as shown in **Figure 5A**).

Our results have specifically shown that a herd immunity level of less than 60% before the cessation of OPV led to the failure of poliovirus eradication under typical epidemiological parameters adopted in this paper. This suggests that maintaining more than 90% OPV coverage is not enough to ensure successful eradication, and that every effort should be made to increase the seroconversion rate in developing countries. Another important parameter affecting the probability of eradication is the recovery rate γ estimated from the mean infectious period. Most data concerning virus excretion rates available from field studies were for the type 1 vaccine strain (Alexander et al., 1997), while much less information is available for types 2 and 3. As type 2 and particularly type 3 have longer excretion periods than type 1, these strains are

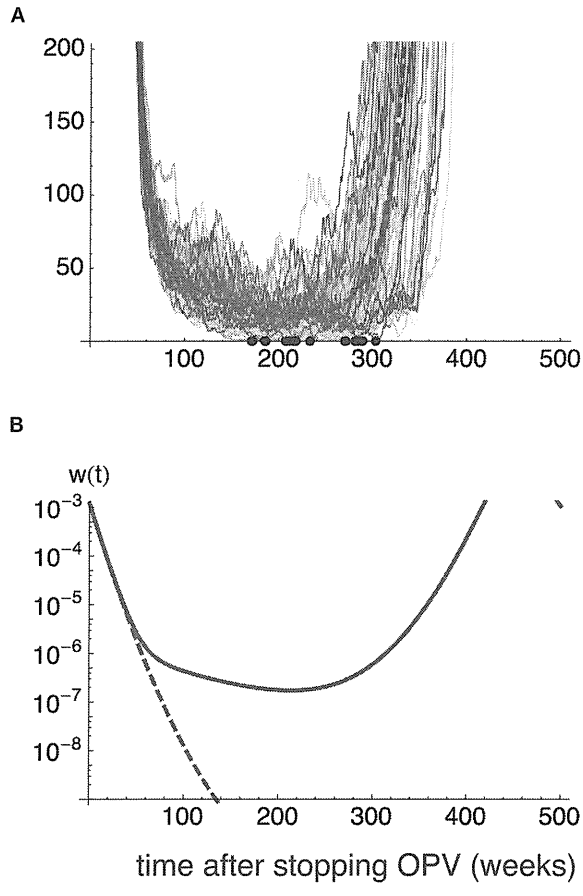

FIGURE 8 | The effect of a high-risk group on global eradication. One in 10,000 (0.01%) of hosts are assumed to be born having a longer excretion period (lower recovery rate γ') when infected by virus. Remaining hosts have the recovery rate γ. $\gamma' = 0.1\gamma$ and $\gamma = 0.2$ is assumed and values $p = 0.7$, $\beta = 2.5$, $\mu = 0.1$, $u = 0.00025$, $K = 10^8$ are used for other parameters. Without the high-risk group, i.e., when all hosts have the recovery rate $\gamma = 0.2$, global eradication is certain. However, with the addition of a fraction 0.01% of high-risk group in the population, eradication fails in 79 out of 100 independent runs, allowing the outbreak of virulent virus. **(A)** Sample paths for the number of infecteds for 100 independent runs. Thick broken lines show the deterministic trajectory. **(B)** Deterministic trajectories for the fraction of infected $w(t)$ when all hosts have the recovery rate γ (broken line), and when 0.01% of hosts have lower recovery rate γ' (solid line).

more likely to persist after cessation of OPV and be the causative agents of outbreaks. In assessing risk, we varied the recovery rate in the range $\gamma = 0.1 - 0.25$, based on estimates for the excretion period of type 3 poliovirus, which appears to have the longest excretion period. Whether this overestimates the risk will eventually be settled by more accurate estimations of excretion periods. However, there may not be enough time to allow the necessary studies, and action may need to taken now assuming the worst possible scenario.

We have shown that even when the mean infectious period is far below the fatal level for eradication failure (e.g., less than 7 weeks in the example shown in **Figure 5B**), the presence of a tiny fraction of immunodeficient individuals greatly increases the risk of disease re-emergence. This was because the primary immunodeficient group acts as a long-term viral reservoir, allowing the virus to persist through the endangered period around t_c (which comes typically 150–200 weeks after the cessation of OPV). At present, no evidence exists whether secondary immunodeficient groups, such as HIV infected patients, could act as a long-term reservoir of poliovirus, but it is possible. Monitoring virus excretion from such high-risk groups would become critically important.

Another factor that drastically increases the risk of polio outbreak after the cessation of OPV is lower transmission rate β_a of attenuated viruses than that β_v of vaccine-derived virulent viruses, as we have shown in **Figure 5** where the results for $\beta_a = \beta_v/2$ is compared with the case $\beta_a = \beta_v$. If we further reduces the transmission rate of attenuated viruses to $\beta_a = \beta_v/4$, the risk of outbreak rises up still more (not shown). This rather unexpected and hazardous dependency comes from the fact that silent circulation of attenuated viruses under vaccination is beneficial in increasing the efficiency of herd immunity. The more is the transmission rate of attenuated viruses, the less is the fraction of hosts that remain susceptible under a fixed vaccination rate. Reducing the transmission rate of attenuated viruses thus increases the susceptible density under vaccination, and hence shortens the time until the susceptible density hits the epidemiological threshold after the cessation of OPV.

Transmission rates (β) can be estimated from R_0, which in turn have been estimated from the mean host age at infection (Anderson and May, 1982; Patriarca et al., 1997; Fine and Carneiro, 1999). Such surveys indicate that R_0 of vaccine-derived poliovirus lies in the range 5–25, depending on the hygiene levels of the region. This is well above the threshold $R_0 = 1$ that allows circulation in susceptible hosts. Eradication probability can be increased by reducing the transmission rate, i.e., by preventing vaccine-derived viruses from circulating in the population as much as possible. Public health attempts to reduce contact with infectious individuals becomes important in reducing the transmission rate β. At the same time, monitoring the circulation of shed virus in the healthy human population and environment becomes even more important after the last round of OPV.

Many studies have shown that immunity by IPV cannot prevent re-infection by poliovirus (Murdin et al., 1996). However, IPV immunization reduces mean excretion duration by 40% compared to unimmunized cases, thus increasing the recovery rate γ by 67% (Henry et al., 1966). IPV also reduces the transmission rate because the number of excreted viruses per unit time also declines. As a result of the increased γ and decreased β, the probability of eradication is higher if IPV immunization follows the cessation of OPV than if no program follows it. Although eradication cannot be achieved without OPV, IPV should be considered, together with its high seroconversion rate, as the primary follow-up strategy after OPV cessation to prevent the secondary transmission of vaccine-derived virus (Ghendon and Robertson, 1994; Sutter et al., 2000).

Neither escape-mutation by antigenic drift (Nowak and May, 1991; Nowak et al., 1991; Sasaki, 1994; Haraguchi and Sasaki, 1997; Sasaki and Haraguchi, 2000) nor the emergence of vaccine-resistant strains (Anderson and May, 1991; McLean,

1995) is considered in this paper, though, in our analysis of IPV immunization, both attenuated and virulent viruses can be regarded as IPV-resistant strains. The presence of multiple serotypes in the viral population complicates the eradication strategy (Lipsitch, 1997). The reason we have ignored such factors in this model of polio eradication is the observation that nucleotide divergence within the VP1 region, which includes the antigenic site, is less than 1.4% in vaccine strains, enabling the protection by OPV or IPV immunization (Matsuura et al., 2000). In a study using a monoclonal antibody toward a vaccine strain, substitutions in the VP1 region did affect neutralization (Wiegers et al., 1989). However, these vaccine-derived strains could still be neutralized by polyclonal antiserum (Matsuura et al., 2000), or be prevented under well-maintained herd immunity (Iwai et al., 2008).

Our model suggests that susceptible host density exceeds the threshold around the time $t_c \approx Lp/R_0$ after the cessation of OPV (e.g., $t_c = 140$ weeks when life expectancy $L = 1/u = 4000$ weeks, immunization fraction $p = 0.7$ and basic reproductive ratio $R_0 = 20$). During the dangerous period around t_c, additional surveillance systems other than normal AFP (acute flaccid paralysis) surveillance should be organized to reduce the risk of re-emergence:

1. Seroepidemiological surveillance of the seroconversion rate within a population. For communities with low seroconversion rates, additional immunization by IPV should be offered. Herd immunity should be maintained at a level over 80% seroconversion.
2. Surveillance of the environment and of shed virus from the source of infection. Upon poliovirus isolation, immunization by IPV is to be administrated to the risk area.
3. Public health administration. A hygiene control program (hand washing practice, use of disposal diapers, etc.) would contribute to the reduction in transmission rate β, preventing the virus from circulating.
4. Monitoring of high-risk groups such as immunodeficient individuals. It is very difficult to use IPV globally due to economic reasons and other administrative difficulties. IPV immunization in restricted regions and in at-risk communities, together with good surveillance systems and hygiene control programs, would be more practical tactics to globally extinguish vaccine-derived viruses.

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APPENDIX

DERIVATION OF EQ. 9

Here we derive Eq. 9 in the text. This is derived by noting that there may be i infected hosts in the next time step either if an infected host gives rise to $i-1$ secondary infections and itself remains infected, or if it gives rise to i secondary infections and itself dies or recovers. Thus

$$\begin{aligned} q(t) &= (1-\delta)\sum_{i=1}^{\infty}\frac{\lambda(t)^{i-1}}{(i-1)!}e^{-\lambda(t)}q(t+1)^i \\ &\quad + \delta\sum_{i=0}^{\infty}\frac{\lambda(t)^i}{i!}e^{-\lambda(t)}q(t+1)^i \\ &= \left[(1-\delta)q(t+1)+\delta\right]e^{-\lambda(t)(1-q(t+1))} \\ &\quad \times\sum_{j=0}^{\infty}\frac{\left\{\lambda(t)q(t+1)\right\}^j}{j!}e^{-\lambda(t)q(t+1)} \\ &= \left[(1-\delta)q(t+1)+\delta\right]e^{-\lambda(t)(1-q(t+1))} \end{aligned} \tag{A1}$$

with $\lambda(t)=\beta K x(t)$, which then leads to (9) in the text.

APPROXIMATE TIME AND NUMBER OF INFECTEDS AT THE MINIMUM POINT

It is useful to obtain an explicit formula for the minimum number of infecteds and the time at which this number reaches its minimum in the deterministic trajectory. This clarifies the parameter dependence on the risk of re-emergence. We found the following approximation useful. We ignore the first term in the right hand of (8a), because it remains very small during the time interval from $t=0$ to $t=t_c$, to give

$$x(t) = 1-(1-x_0)e^{-ut}, \tag{A2}$$

(see, for example, Anderson and May, 1991). Integrating (8b) we have

$$w(t) = w_0 exp\left[\int_0^t \left[\beta x(s)-(u+\gamma)\right]ds\right]. \tag{A3}$$

Clearly $w(t)$ attains the local minimum when $t=t_c$ where $\beta x(t)=u+\gamma$. Letting

$$a=\frac{\beta-(u+\gamma)}{u}=k(R_0-1), \quad b=\frac{\beta(1-x_0)}{u}=kR_0(1-x_0), \tag{A4}$$

with $k=(u+\gamma)/u$ and $R_0=\beta/(u+\gamma)$, we therefore have

$$t_c \approx \frac{1}{u}log\left[\frac{b}{a}\right]=Llog\left[\frac{R_0(1-x_0)}{R_0-1}\right], \tag{A5a}$$

$$w_c \approx w_0\left(\frac{b}{a}\right)^a e^{a-b}=w_0\left(\frac{R_0(1-x_0)}{R_0-1}\right)^{k(R_0-1)} exp\left[R_0x_0-1\right], \tag{A5b}$$

where $L=1/u$ is the life expectancy, and $R_0=\beta/(u+\gamma)$ the basic reproductive rate. We expect a high probability of eradication if Kw_c is sufficiently smaller than 1, and show significant risk of re-emergence if it is 10 or more. The deviation of w_c from the true minimum is small in logarithmic scale, though it is as large as 50% in normal scale. However, for the purpose of quickly checking the likelihood of successful eradication, this formula is useful. If we assume that x_0 and w_0 take the values at the endemic equilibrium with the vaccination rate p (Eq. 5 in the text), we obtain the asymptotic formula for large R_0:

$$t_c \approx Lp/R_0, \quad (R_0>>1), \tag{A6a}$$

$$Kw_c \approx K\frac{D}{L}exp\left[-\frac{p^2}{2R_0}\frac{L}{D}\right], \quad (R_0>>1,\ L>>D), \tag{A6b}$$

where $D=1/\gamma$ is the mean duration of infection.

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ORIGINAL ARTICLE

Longitudinal surveillance of *Haemophilus influenzae* isolates from pediatric patients with meningitis throughout Japan, 2000–2011

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Abstract In Japan, β-lactamase-nonproducing, ampicillin-resistant organisms have been evident among *Haemophilus influenzae* type b (Hib) isolates since 2000, when no appropriate vaccine had been approved. We therefore performed molecular analysis of agents causing *H. influenzae* meningitis nationwide over the following 10 years. Some 285 institutions have participated in surveillance since 2000. The capsular type and resistance genes of 1,353 isolates and 23 cerebrospinal fluid samples from pediatric patients with meningitis we had received from 2000 to 2011 were analyzed by polymerase chain reaction. Blood and spinal fluid test results obtained when patients were admitted were examined for correlation with outcomes. Hib was found in 98.9 % of isolates. We received more than 100 Hib isolates per year until vaccination began in December 2008, when these isolates decreased, especially since establishment of a special fund to promote vaccination in November 2010. Decreased incidence among infants 7 months to 2 years old has been particularly notable. However, the rate of ampicillin-resistant organisms has increased to more than 60 % of all isolates since 2009. We received 587 replies to a questionnaire concerning outcomes, indicating 2 % mortality and 17.7 % serious morbidity. Age of 6 months or younger and presence of disseminated intravascular coagulation at admission were related to an unfavorable outcome ($p < 0.05$), but ampicillin resistance was not. Combination therapy with third-generation cephem and carbapenem agents was used initially for 72 % of patients. Routine immunization can prevent Hib meningitis in children.

Keywords *Haemophilus influenzae* type b (Hib) · Genotypic β-lactamase-nonproducing (gBLNAR) · Polymerase chain reaction · Surveillance · Molecular epidemiology

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Introduction

Community-acquired bacterial meningitis in children is a serious infection that occasionally is fatal. Pathogens and infection rate differ according to patient age; availability of vaccination against *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, and others; and location in a developed versus a developing country.

Hib is well known to cause meningitis, epiglottitis, purulent arthritis, pericarditis, pneumonia, and other infections in infants and children over 3 months of age. Based on data from our surveillance [1] and from Ishiwada et al. [2], the incidence of Hib is approximately 10 to 12 per 100,000 children under 5 years of age. However, Hib meningitis already is uncommon in many countries where Hib vaccination has been introduced. Unfortunately, Hib vaccine was not approved by the Japanese Ministry of Health, Labour and Welfare until January 2007, and voluntary vaccination of children only began in late 2008. In November 2010, vaccination of children with Hib and

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heptavalent pneumococcal conjugate vaccine (PCV7) was recommended by the Provisional Special Fund for the Urgent Promotion of Vaccination against Such Diseases as Cervical Cancer. The vaccination rate among infants at risk throughout Japan is estimated to be 40–60 % as of the end of 2011.

Meanwhile, the rate of β-lactamase-nonproducing, ampicillin (AMP)-resistant *H. influenzae* (BLNAR) and Hib isolates from patients with meningitis have increased rapidly in parallel with their exponential increase in patients with respiratory tract infections (RTI) [1, 3, 4]. Although BLNAR strains showing decreased affinity for β-lactam antibiotics were first described in the United States in the 1980s [5, 6], these strains have remained rare in that country [7, 8] and in the European Union (EU) [9–11], except for France.

The resistance mechanism in BLNAR involves mutations in the *ftsI* gene encoding penicillin-binding protein 3 (PBP3), which mediates septal peptidoglycan synthesis in the cell wall. PBP3 is the main target of cephalosporin antibiotics, which differs from that of penicillins and carbapenems. Accordingly, susceptibility to cephalosporin clearly is affected by *ftsI* gene mutations [12]. We have identified amino acid substitutions at three PBP3 positions mainly associated with decreased β-lactam susceptibility: Asn526Lys, Arg517His, and Ser385Thr. Strains with substitutions of either Asn526Lys or Arg517His and also Ser385Thr were classified as genotypic BLNAR (gBLNAR) based on correlations with β-lactam susceptibility. Other strains with only Asn526Lys or Arg517His substitutions were classified as genotypic Low-BLNAR (gLow-BLNAR). In Australia [13], France [14], and Norway [15], the incidence of gLow-BLNAR isolates possessing an Asn526Lys substitution with AAA sequences was significant, but not in Japan, where AAG sequences contributed to this substitution.

In this report, we describe results in *H. influenzae* isolates from meningitis patients collected by the Nationwide Surveillance for Bacterial Meningitis (NSBM) working group and impact on survival outcome and presence or absence of sequelae for the following: yearly changes in genotypic β-lactam resistance, blood and spinal fluid test results, and antibiotics initially used at disease onset.

Materials and methods

Patients and strains

A total of 1,353 *H. influenzae* strains isolated from cerebrospinal fluid (CSF) collected from pediatric patients with bacterial meningitis were sent to Kitasato Institute for Life Sciences from clinical laboratories at 285 Japanese medical institutions between January 2000 and December 2011. CSF samples sent by pediatricians for identification of the causative pathogen also included 23 samples containing *H. influenzae* DNA. These strains and clinical samples were sent to our laboratory accompanied by two documents that protected the anonymity of the patient: one is a record of the informed consent obtained from the guardians of the infants and children, and the other is a survey form that was filled out by the attending physician.

Genotypic determination of β-lactam resistance was performed immediately by polymerase chain reaction (PCR) on all isolates received to determine *ftsI* gene mutations as described in the following section. These PCR results were immediately reported to the referring pediatrician and the laboratory technicians.

Polymerase chain reaction

Conventional PCR [16] was performed on *H. influenzae* isolates using six sets of primers that we had constructed for routine use in our laboratory: P6 primers to amplify the *p6* gene for identification of the *H. influenzae* species; TEM-1 primers to amplify a part of the TEM-1-type β-lactamase gene (bla_{TEM}); ROB-1 primers to amplify a part of the ROB-1-type β-lactamase gene (bla_{ROB}); PBP3-S primers to identify an Asn526Lys amino acid substitution in the *ftsI* gene; PBP3-BLN primers to identify Asn526Lys and Ser385Thr amino acid substitutions in the *ftsI* gene; and serotype b primers to amplify a part of the Hib-specific *capB* locus. PCR cycling conditions were 35 cycles at 94 °C for 15 s; 53 °C for 15 s, and 72 °C for 15 s.

Thereafter, Asn526Lys and Ser385Thr amino acid substitutions were separately identified by the real-time PCR method we constructed in 2007 [17].

Isolates suspected to have an Arg517His substitution based on susceptibility to ampicillin (AMP) and cefotaxime (CTX) were subjected to direct sequencing to detect this substitution, because useful primers could not be designed.

Genotypic resistance patterns were classified as follows: gBLNAS, without any of the three substitutions; gBLPAR, producing β-lactamase TEM-1 or ROB-1; gLow-BLNAR, with substitution of Asn526Lys or Arg517His; gBLNAR, with two or three substitutions, Asn526Lys or Arg517His, as well as Ser385Thr; gBLPACR-I, producing β-lactamase but having a gLow-BLNAR genotype; and gBLPACR-II, also producing β-lactamase but having a gBLNAR genotype.

Statistical analysis

We used Microsoft Excel 2010 for Statistics (SSRI, Tokyo, Japan) and Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA) for data analysis. Categorical variables were compared using chi-squared tests. Continuous variables were compared using Student's t test. A p value less than 0.05 indicated a significant difference between groups.

Results

Changes in resistance among strains for year to year

A breakdown of Hib and nontypeable *H. influenzae* (NTHi) among 1,353 isolates and 23 spinal fluid samples collected from pediatric inpatients with *H. influenzae* meningitis during the study period is shown in Table 1. Among all isolates, 98.9 % were identified to be serotype b; the remaining 1.1 % represented NTHi. One quarter of spinal fluid samples were shown to contain Hib DNA by real-time PCR. No other serotypes were recognized.

Figure 1 shows year-to-year changes in β-lactam resistance among strains. Resistance was identified molecularly by conventional PCR (from 2000 to 2009) and by real-time PCR (from 2010 to 2011) for the *ftsI* gene encoding PBP3, the bla_{TEM} gene encoding TEM-1 β-lactamase, and the bla_{ROB} gene encoding ROB-1 β-lactamase, respectively [16, 17]. For strains that showed discrepancies between their susceptibility for AMP or CTX and the results of PCR, the *ftsI* gene was analyzed by sequencing.

As shown in Fig. 1, Hib gBLNAR first was identified as a novel resistant strain in 2000. Since then, the resistance rate has increased exponentially over time, exceeding 60 % in 2009 and reaching approximately 70 % in 2011. Over the same interval, gBLNAS and gBLPAR, respectively, decreased from 32 and 26 % in 2000 to 8 and 0 % in 2011.

Distributions of patient age and β-lactam resistance by year

Yearly distribution of patient age and β-lactam resistance according to genotypic identification is shown in Fig. 2. Hib vaccination of children began on a voluntary basis on December 19, 2008. Subsequently, the immunization rate for Hib in Japanese children up to 1 year old is estimated to have been approximately 10 % in 2009, 20 % in 2010, and 50–60 % in 2011, representing an increase every year (data not shown here).

Although longitudinal surveillance demonstrated that the largest number of patients up to 1 year old continued until 2008, these patients decreased beginning in 2009 when Hib vaccination started. In 2011, the total of cases decreased dramatically to 46, about half the usual collected strains. Further, no differences in prevalence of gBLNAR were seen between age groups.

Details of sequelae

Details of sequelae in patients with *H. influenzae* meningitis are listed in Table 2.

This information was obtained from the questionnaires completed by attending physicians. Among the 655 responses, details concerning patients with or without sequelae were recorded in 587 cases. Death was reported in 12 patients (2.0 %), whereas serious sequelae, mainly including brain atrophy or infarction, motor dysfunction, and auditory or visual dysfunction, were noted in 104 (17.7 %).

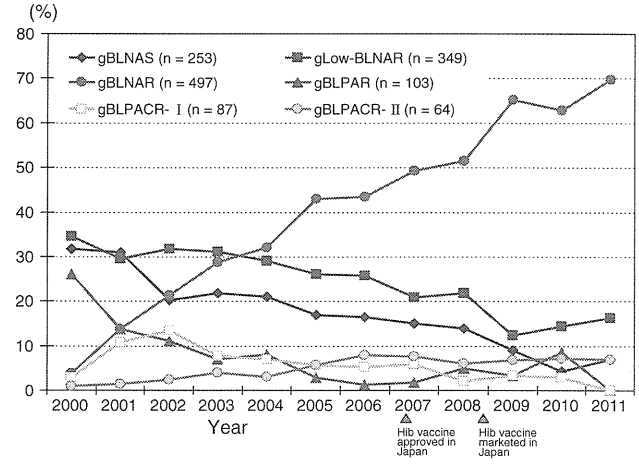

Fig. 1 Year-to-year changes in genotypically classified resistance types among strains isolated between 2000 and 2011. *g* genotype; *Hib* type b *Haemophilus influenzae*; *gBLNAS* β-lactamase-nonproducing, ampicillin (AMP) susceptible *H. influenzae*; *gBLNAR* β-lactamase-nonproducing, AMP-resistant *H. influenzae*; *gLow-BLNAR* β-lactamase-nonproducing, low-level AMP-resistant *H. influenzae*; *gBLPAR* TEM-1 β-lactamase-producing, AMP-resistant *H. influenzae*; *gBLPACR* TEM-1 β-lactamase-producing, amoxicillin/clavulanic acid-resistant *H. influenzae*

Table 1 Strains and samples isolated from pediatric patients throughout Japan with *Haemophilus influenzae* meningitis, by year

Samples	Serotype	Years												
		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	Total
Strain (n = 1,353)	Hib	104	138	162	129	99	141	150	120	99	89	68	39	1,338
	NTHi		1	2		1	1	2		2		2	4	15
Spinal fluid (n = 23)[a]	Hib						1	1	1	1	2			6
Unknown				2	2	1		1	1		5	2	3	17
Total		104	139	166	131	101	143	154	122	102	96	72	46	1,376

Hib type b *Haemophilus influenzae*, *NTHi* nontypeable *Haemophilus influenzae*

[a] Samples were analyzed by real-time PCR to detect the *capB* gene encoding capsular type b polysaccharide by a method in our laboratory [3]

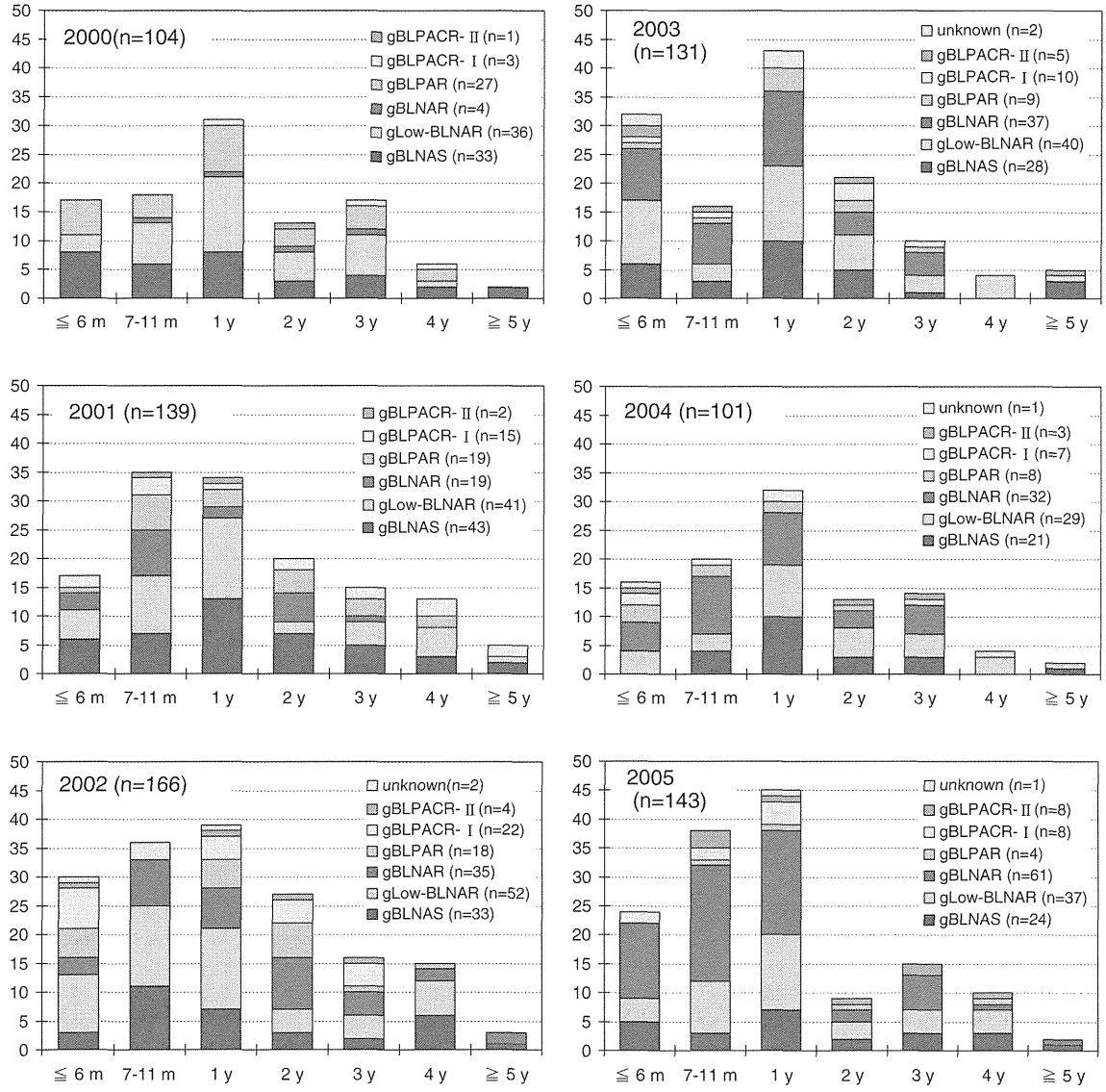

Fig. 2 Year-to-year distribution shows relationships between patient age and genotypically classified β-lactam resistance types among strains isolated between 1999 and 2011

Characteristics of patients with and without sequelae

Table 3 compares characteristics of patients with versus without sequelae after the onset of Hib meningitis. Although sequelae were more frequent in patients 6 months old or younger at the time of onset, significant differences in onset age were observed between the two groups ($p < 0.05$). The presence of disseminated intravascular coagulation (DIC) also significantly affected sequelae.

No significant differences were noted in blood test and spinal fluid test results. Outcome also was not affected by resistance type of the Hib pathogen, namely, whether gBLNAR or not.

Correlation between antimicrobial choice and outcome

Relationships between initial antimicrobial therapy given to meningitis patients on hospital admission and outcomes are shown in Fig. 3. Half the patients ($n = 305$, 52.3 %) received initial therapy with combinations of a third-generation cephem and a carbapenem agent, namely, CTX and meripenem (MEM) or panipenem (PAM), or ceftriaxone (CRO) and MEM or PAM. The next most frequent treatment was AMP and CTX or CRO therapy (18.3 %). Monotherapy with CTX or CRO was given only to 12.7 %.

When the causative agent was identified as *H. influenzae*, the therapeutic regimen was changed to combination

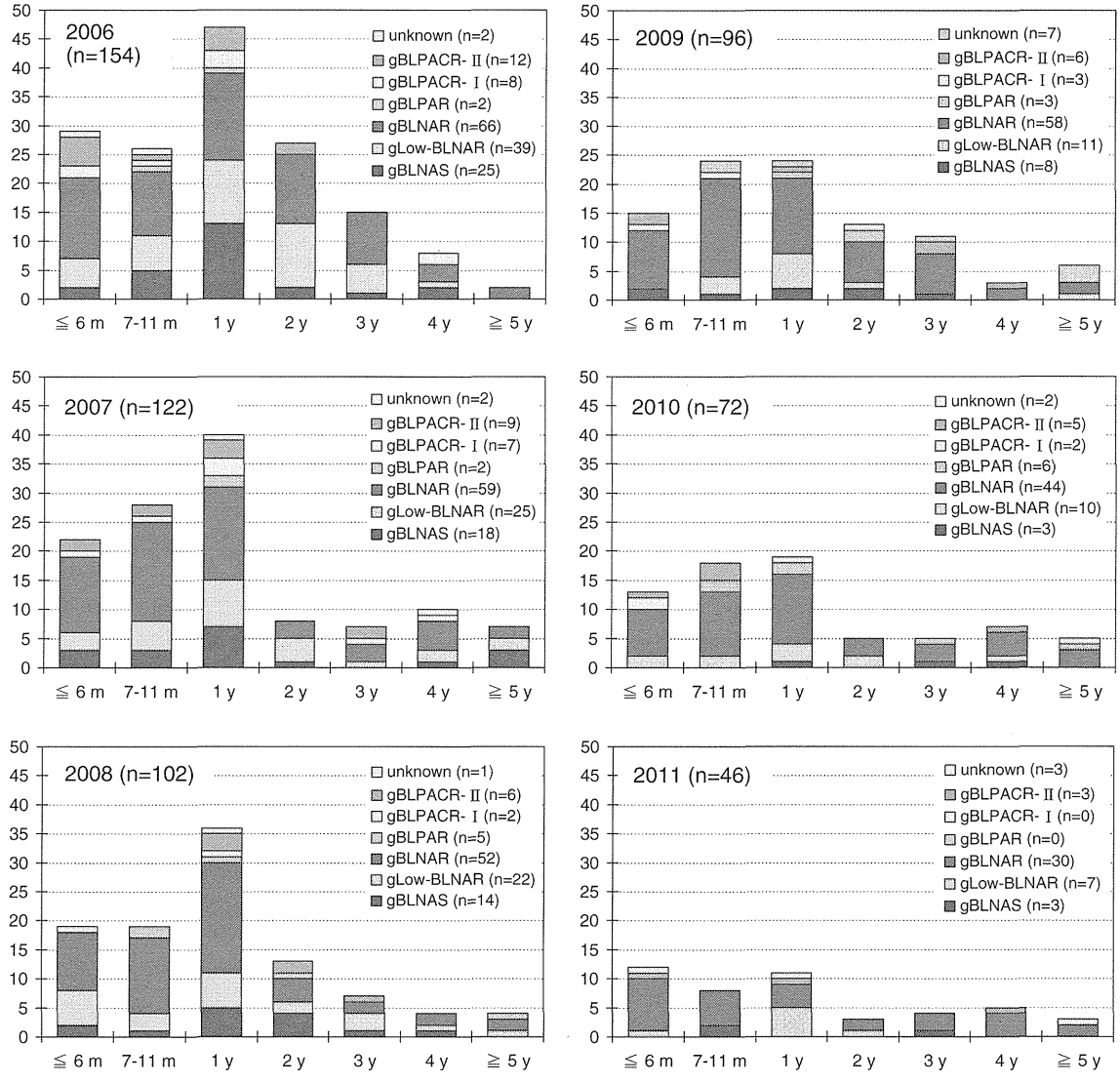

Fig. 2 continued

therapy. A significant relationship between antimicrobial choice and outcome was not observed.

Discussion

Bacterial meningitis is an infectious disease with a high occurrence rate of fatalities and serious sequelae in children. Accordingly, vaccine development has been attempted for many years. The Hib conjugate vaccine was developed to prevent occurrence of bacterial meningitis and severe infections caused by capsular type b *H. influenzae*, the most frequent causative organism. In the United States, Hib vaccine was licensed for use in children in 1987 and entered the standard vaccination schedule in 1990 [18, 19]. As a result, a dramatic decrease in bacterial meningitis from Hib became evident after a few years [19–21]. This vaccination then was introduced in many other countries, where Hib infection now has become a largely eradicated disease.

In Japan, introduction of Hib vaccine was much delayed, for reasons including a low incidence of bacterial meningitis from Hib relative to other countries; high accessibility of Japanese medical institutions; easily used antibiotics; and lack of a feeling of urgency concerning vaccination.

During the decade preceding Japanese adoption of the vaccine, gBLNAR, a new resistant strain, emerged among Hib isolates [1]. These resistant bacteria increased rapidly, causing a major therapeutic problem. Previously, 22–26 % of Hib isolates were β-lactamase-producing strains [1, 3], so the first-choice agent was a third-generation cephem, CTX [22]. To address the problem of gBLNAR, treatment shifted heavily to combination therapy with a third-generation cephem and carbapenem [22], based on the

mechanism of resistance in these strains. The targets of β-lactam antibiotics are the penicillin-binding proteins (PBPs) involved in peptidoglycan synthesis; in BLNAR, the *ftsI* gene encoding PBP3 shows a number of important mutations [12].

Table 2 Sequelae following *Haemophilus influenzae* meningitis

	Number of patients (% of total)
Sequelae (+)	116 (19.8)
Death	12 (2.0)
Brain death	4 (0.7)
Cerebral palsy	1 (0.2)
Hydrocephalus	5 (0.9)
Brain atrophy/brain infarct	22 (3.7)
Epilepsy	5 (0.9)
Motor dysfunction	24 (4.1)
Auditory dysfunction	12 (2.0)
Visual disorder	2 (0.3)
Other[a]	31 (5.3)
Sequelae (−)	471 (80.2)
Total	587

[a] Includes two patients with two sequelae

Therefore, BLNAR susceptibility to cephems, which target mainly PBP3, is 50–100 times decreased compared to that of susceptible organisms. However, because the main target of carbapenems is unrelated to PBP3, susceptibility of BLNAR to carbapenem is not affected [3]. A synergistic effect from a combination of two kinds of agents with their different target sites is expected.

The Hib vaccine was approved in Japan in January 26, 2007, and has been marketed since December 19, 2008. Accurate nationwide vaccination numbers are not available, but the estimated vaccination rate in the infant population (up to 1 year of age), based on numbers of vials delivered, was 10 % in 2009, 20 % in 2010, and 40–60 % in 2011. The high vaccination rate in 2011 is attributable largely to official support provided by the Provisional Special Fund for the Urgent Promotion of Vaccination Against Such Diseases as Cervical Cancer. This initiative includes Hib and PCV7 vaccination of infants.

Incidence of Hib meningitis among children 7 months to 1 year of age has decreased gradually since 2009 in our results, reflecting the effect achieved by Hib vaccination. However, the decrease in incidence among infants under 6 months of age is less evident than that among those between 7 months and 3 years old. Infants vaccinated at such a young age may have difficulty producing antibody titers sufficient to prevent Hib infection at that stage.

Table 3 Characteristics of children with Hib meningitis with and without sequelae

Characteristics	Sequelae (+) (n = 116)	Sequelae (−) (n = 471)	p value
Age			
≤6 months	28 (24.1 %)	77 (16.3 %)	*0.049*
7–11 months	24 (20.7 %)	106 (22.5 %)	0.673
1 year	32 (27.6 %)	118 (25.1 %)	0.575
>2 years	32 (27.6 %)	170 (36.1 %)	0.084
Underlying disease (+/−)	12/95 (11.2 %)	46/389 (10.6 %)	0.847
Initially seen at an other hospital (+/−)	74/27 (73.3 %)	327/96 (77.3 %)	0.389
DIC (+/−)	31/80 (27.9 %)	37/414 (8.2 %)	*<0.001*
Blood values			
WBC (cell/μl)	9,150[a] (5,397–14,375)[b]	11,200 (7,100–16,720)	0.562
PLT (10^4/μl)	18.4 (8.9–31.3)	21.2 (13.8–31.7)	0.656
CRP (mg/dl)	15.4 (7.3–22.8)	14.0 (7.5–20.8)	0.587
Spinal fluid values			
Cells (cell/μl)	6,176[a] (2,718–11,170)[b]	7,872 (3,375–14,720)	0.235
Glucose (mg/dl)	21 (5–42)	31 (10–51)	0.342
Protein (mg/dl)	159 (98–240)	142 (96–212)	0.762
Steroid therapy (+/−)	88/8 (91.7 %)	384/25 (93.9 %)	0.428
gBLNAR + gBLPACR II	41(35.3 %)	166 (35.2 %)	0.983

Fifty percent of all subjects included in the range

DIC disseminated intravascular coagulation, *PLT* platelets, *CRP* C-reactive protein, *gBLNAR* genotypic ampicillin (AMP)-resistant *H. influenzae*, *gBLPACR* genotypic TEM-1 β-lactamase-producing, amoxicillin/clavulanic acid-resistant *H. influenzae*

[a] Median value analyzed by box-and-whisker plots

[b] Value shown in parentheses is 25 percentile and 75 percentile analyzed by box-and-whisker plots

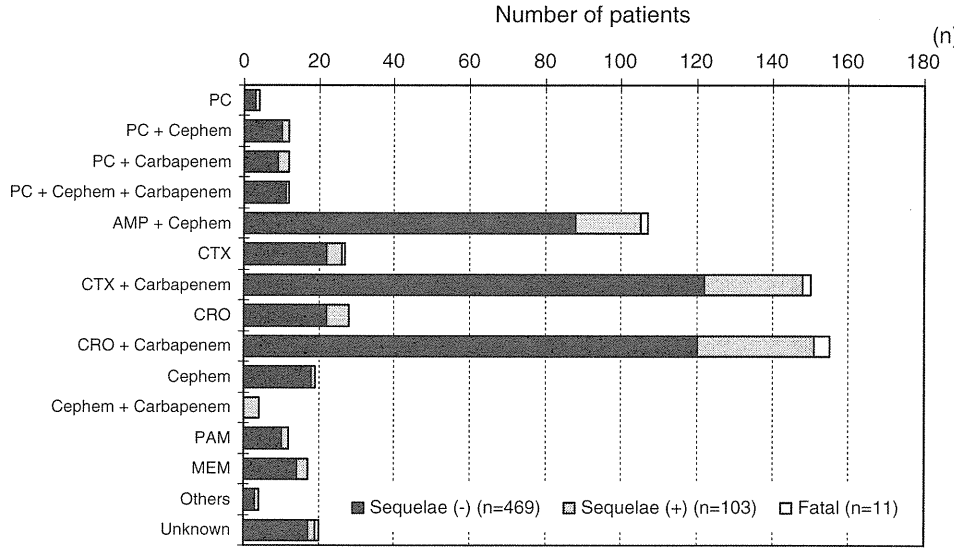

Fig. 3 Correlation between initial antimicrobial therapy given to meningitis patients upon hospitalization and outcomes. *PC* piperacillin (PIPC) and PIPC/tazobactam, *AMP* ampicillin, *CTX* cefotaxime, *CRO* ceftriaxone, *PAM* panipenem, *MEM* meropenem

Continued surveillance is needed to see how the incidence of Hib meningitis may change in the future.

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Application of the Real-Time PCR Method for Genotypic Identification of β-Lactam Resistance in Isolates from Invasive Pneumococcal Diseases

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We sought to identify genotypic resistance classes by real-time PCR in 300 *Streptococcus pneumoniae* isolates from invasive pneumococcal diseases. Primers and molecular beacon probes were designed for the *lytA* gene, 3 *pbp* genes, and the *mef*A/*erm*B genes. Targeted sequences of *pbp1a*, *pbp2x*, and *pbp2b* genes in susceptible strain R6 corresponded to those of penicillin G-nonsusceptible strains, including sites within or adjacent to conserved amino acid motifs. If amplification did not occur, the corresponding penicillin-binding protein (PBP) was considered to possess amino acid substitution(s) affecting minimal inhibitory concentrations (MICs) of β-lactam antibiotics. Real-time PCR required 90 min or less. Strains were assigned to six genotypic classes: Genotypic penicillin-susceptible *S. pneumoniae* (gPSSP) with 3 normal genes (22.3%); genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*) with an abnormal *pbp2x* gene (25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene (7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes (11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes (4.7%); or genotypic penicillin-resistant *S. pneumoniae* (gPRSP) with 3 abnormal PBP genes (29.0%). Sensitivity and specificity of real-time PCR compared with those of conventional PCR were high, 73.7–100% and 97.7–100%, respectively. As for relationships between genotype and β-lactam MICs, 90% of MICs for every resistance class were distributed within three serial dilutions for almost all antibiotics. MICs of each β-lactam antibiotic were estimated with high probability from genotypic patterns. In conclusion, determination of genotypic classes of *S. pneumoniae* using rapid real-time PCR is useful in selecting effective therapeutic agents for patients with pneumococcal infection.

Introduction

Streptococcus pneumoniae is a leading etiologic agent in children and adults with severe invasive infections that contribute importantly to morbidity and mortality.[20,32] Strains resistant to penicillin G (PEN) have emerged and spread rapidly worldwide.[1,15]

In Japan, clinical isolates of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) have increased rapidly since the late 1990s among school and preschool children as well as patients aged 65 years or older with either respiratory tract infections (RTI) or invasive pneumococcal diseases (IPD).[28,30] The mortality rate reportedly is higher in elderly IPD patients than in pediatric patients.[8]

Characteristically, PRSP and PISP strains show simultaneous resistance to cephalosporin antibiotics used in ambulatory practice.[30] The resistance mechanism for β-lactam antibiotics in PRSP and PISP is a decrease in affinities of three PEN-binding proteins (PBP) involved in peptidoglycan synthesis. These three enzymes, PBP1A, PBP2X, and PBP2B, are encoded by the *pbp1a*, *pbp2x*, and *pbp2b* genes, respectively. Among PEN-nonsusceptible strains (PRSP and PISP), abnormal genetic mosaic patterns of *pbp1a*, *pbp2x*, and/or *pbp2b* were found to differ from those of PEN-susceptible *S. pneumoniae* (PSSP).[9,14] Although a variety of mosaic regions have been detected in each gene, the main contributors to β-lactam resistance are amino acid substitutions identified within or adjacent to conserved amino acid motifs such as Ser-Thr-Met-Lys (STMK), Ser-Ser-Asn (SSN), and Lys-Ser-Gly (KSG).[2,3,13,23,24]

Therefore, we established a conventional PCR method to determine whether or not a pneumococcal isolate is PEN-susceptible according to molecular evidence.[31] This PCR was completed within 2.5 hr from selection of a colony for testing by amplification and gel electrophoresis. The resistance pattern based on the results of conventional PCR

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was defined as genotypic (g) resistance and represented by designations such as gPRSP (*pbp1a+pbp2x+pbp2b*), gPISP(*pbp1 a+pbp2x*), gPISP(*pbp2x*), and gPSSP. Currently, the prevalence of gPRSP possessing three abnormal *pbp* genes exceeds 46% among pediatric patients and 17% among adults in Japan.[8]

Given this situation, therapeutic choices for Japanese IPD patients have gradually eroded, with empirical first-line therapy shifting from penicillins or third-generation cephalosporins to carbapenem antibiotics. At the same time, numbers of adults and elderly persons with various underlying diseases posing high risk of IPD have increased rapidly.

In the present study, we aimed to construct a novel assay using real-time PCR that eliminates the need for gel electrophoresis, allowing completion of all procedures within 90 min. We describe sensitivity and specificity of our real-time PCR compared with conventional PCR and efforts to estimate MICs of therapeutic agents against various strains belonging to different PBP genotypic classes.

Materials and Methods

Strains and serotyping

Clinical isolates of *S. pneumoniae* obtained from IPD patients were collected from 186 clinical laboratories at medical institutions participating in our program of active nationwide surveillance for emerging and re-emerging of infectious diseases. We randomly selected 300 strains as follows: Blood ($n=218$), cerebrospinal fluid ($n=56$), pleural fluid ($n=14$), joint fluid ($n=6$), and others ($n=6$). These strains were sent to our laboratory from August, 2006, to July, 2007, accompanied by application form with a similar format as the Active Bacterial Core Surveillance (ABCs) case report.

The serotypes of all strains were determined in real time by the Quellung reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). The serotypes of these strains were mainly 6B ($n=47$), 12F ($n=28$), 14 ($n=27$), 3 ($n=26$), 4 ($n=22$), 9 ($n=20$), 19F ($n=19$), 23F ($n=18$), 6A/6C ($n=16$), 19A ($n=14$), 15 ($n=12$), and others ($n=51$).

Real-time PCR primers and molecular beacon probes

Sequences of six sets of primers and molecular beacon (MB) probes and amplicon sizes (bp) applied for our real-time PCR are shown in Table 1. Target genes and the DNA amplification positions were the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae*[12]; the *pbp1a* gene detected in susceptible strains,[18] located in the region including a conserved amino acid motif, STMK, corresponding to that of resistant strains; the *pbp2x* gene detected in susceptible strains,[16] located in the region surrounding the STMK motif corresponding to divergent sequences of resistant strains; the *pbp2b* gene detected in susceptible strain,[10] located in the region adjacent the SSN motif; the *mef*A gene

TABLE 1. PRIMERS AND MOLECULAR BEACON PROBES FOR REAL-TIME PCR

Target gene	*Sequence (5′ to 3′)*	*Position*	*Amplicon size (bp)*	*Target amino acid substitution*
Autolysin (*lytA*)				
Sense primer	CAGAATTAGGTTTTTTCTCGC	723–743	188	—
Reverse primer	TAAGAGTTCGATATAAAGGCG	890–910		
Probe	FAM-CGCGATCAGGTCTCAGCA TTCCAACCGCCGATCGCG-BHQ1	809–830		
PBP 1A (*pbp1a*)				
Sense primer	AAACCGCGACTGGGGATCAAC	2037–2057	239	S(T)MK
Reverse primer	GGTTGAGTCCGACCTTGTTT	2275–2256		↓
Probe	FAM-CGCGATCACTGGGATAGGGG CTACTTTGGCGATCGCG-BHQ1	2174–2196		A or S
PBP 2X (*pbp2x*)				
Sense primer	CCAGGTTCCACTATGAAAGTG	1255–1275	197	S(T)(M)K
Reverse primer	ATCCCAACGTTACTTGAGTGT	1451–1431		↓ ↓
Probe	FAM-CGCGATCAGATGCCACGATTC GAGATTGGGGATCGCG-BHQ1	1353–1375		A F
PBP 2B (*pbp2b*)				
Sense primer	CCTATATGGTCCAAACAGCCT	1566–1586	147	SSN(T)
Reverse primer	GGTCAATTCCTGTCGCAGTA	1712–1693		↓
Probe	FAM-CGCGATCTCGGCACCAGCAAT CTAGAGTCTGATCGCG-BHQ1	1626–1648		A or S
Macrolide efflux (*mef*A)				
Sense primer	GGGACCTGCCATTGGTGTGC	180–199	402	—
Reverse primer	CCCAGCTTAGGTATACGTAC	581–562		
Probe	FAM-CGCGATCCCCCAGCACTCAAT GCGGTTACACGATCGCG-BHQ1	359–382		
Adenine methylase (*erm*B)				
Sense primer	CGTACCTTGGATATTCACCG	721–740	224	—
Reverse primer	GTAAACAGTTGACGATATTCTCG	944–922		
Probe	FAM-CGCGATCCCGCCATACCACAG ATGTTCCGATCGCG-BHQ1	852–872		

encoding the efflux protein for 14-membered macrolide (ML) antibiotics[25]; and the *ermB* gene encoding adenine methylase for 14- and 16-membered ML antibiotics.[27]

Primers and MB probes corresponding to *pbp1a*, *pbp2x*, and *pbp2b* genes were designed to amplify the DNA only in susceptible strains. All MB probes were labeled with a fluorescent reporter of 6-carboxyfluorescein (FAM) at the 5′ end and also with a black hole quencher 1 (BHQ-1) at the 3′ end. Reporters and quenchers were connected to stem oligonucleotides.

Real-time PCR conditions

The real-time PCR reaction mixture consisted of 15 μl of 2× real-time PCR Master Mix (Toyobo, Tokyo, Japan), each primer at 0.2 μM, and each MB probe at 0.3 μM. The final volume of the mixture was adjusted to 30 μl by addition of DNase- and RNase-free H_2O. After each reaction mixture was pipetted into a 96-well plate, plates were stored at −30°C until use.

One colony grown on a sheep blood agar plate was picked up and suspended in 30 μl of lysis solution.[29] The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; Perkin-Elmer Cetus, Norwalk, CT) and heat-treated for 5 min at 60°C and for 5 min at 94°C to obtain template DNA. Next, after wells of the frozen real-time PCR reagent were thawed on ice, 2 μl of each template DNA was added to each well. Real-time PCR was performed immediately with a Stratagene Mx3000P (Stratagene, La Jolla, CA). The PCR conditions included an initial DNA denaturation step of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 50°C for 20 sec, and 75°C for 15 sec. The time required from the lyses reaction to completion of real-time PCR was 90 min.

Conventional PCR

Conventional PCR was performed as a control assay for the real-time PCR in the same strains, using a commercially available kit (Wakunaga Pharmaceuticals, Hiroshima, Japan). The right of commercial production for this kit had been transferred to the company from Ubukata et al.[19,30]

Sequencing of pbp *genes with discrepancies between the two PCR methods*

Both the *pbp1a* and *pbp2x* genes in *S. pneumoniae* strains for which a discrepancy in the PCR data was recognized between the conventional and real-time methods were sequenced to identify the amino acid substitution. PCR primers used for analysis were a sense primer for *pbp1a*, 5′-TGGGA TGGATGTTTACACAAATG-3′; a reverse primer for *pbp1a*, 5′-TGTGCTGGTTGAGGATTCTG-3′; a sense primer for *pbp2x*, 5′-TATGAAAAGGATCGTCTGGG-3′; and a reverse primer for *pbp2x*, 5′-AGAGAGTCTTTCATAGCTGAAGC-3′, as described previously.[2,3]

Amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out using a BigDye® Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA). DNA sequencing was performed with an ABI 3130/3130xl genetic analyzer (Applied Biosystems).

Susceptibility testing

MICs of the five β-lactam antibiotics PEN, ampicillin (AMP), cefotaxime (CTX), meropenem (MEM), and panipenem (PAM) were determined by an agar dilution method using Mueller–Hinton II agar (MH, Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinized sheep blood. Bacterial inoculum size and culture conditions were in accordance with a previously described method.[28]

S. pneumoniae ATCC49619 and R6 reference strains were used as quality controls.

Multilocus sequence typing and eBURST analysis

Multilocus sequence typing (MLST) performed for *S. pneumoniae* strains recognized discrepancy in the data of the two PCR methods. MLST and eBURST analysis was performed according to the MLST site (http://spneumoniae.mlst.net/).

Results

Resistant genotypes determined by real-time PCR

Figure 1 shows four patterns from a computer display connected to the real-time PCR instrument shown just after the PCR reaction was completed. Each tested strain was identified as follows: A, as gPSSP by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes; B, as gPISP (*pbp2x*), with only the *pbp2x* gene not amplified; C, as gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified; and D, as gPRSP (*pbp1a*, *pbp2x*, and *pbp2b*), with all 3 *pbp* genes not amplified. With regard to ML resistance, a strain showing DNA amplification for *mef*A and/or *erm*B genes was identified as ML resistant.

Genotypic classification of β-lactam and macrolide resistance

All strains tested were classified into six genotypic categories by real-time PCR for 3 *pbp* genes: gPSSP with three normal genes ($n=67$, 22.3%); gPISP (*pbp2x*) with an abnormal *pbp2x* gene ($n=76$, 25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene ($n=22$, 7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes ($n=34$, 11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes ($n=14$, 4.7%); and gPRSP with three abnormal PBP genes ($n=87$, 29.0%). Strains examined included 106 from pediatric patients (35.3%) and 194 from adult patients (64.7%). Percentages of the strains representing gPRSP accounted for 50.5% of isolates from children and 17.5% of those from adults.

Although detailed data are not shown, ML resistance in these strains was classified into four genotypic categories based on presence or absence of resistance genes: ML-susceptible strains ($n=58$, 19.3%); 14-membered ML-resistant strains possessing an *mef*A gene ($n=79$, 26.3%); 14- and 16-membered ML-resistant strains possessing an *erm*B gene ($n=150$, 50.0%); or an ML-resistant strain possessing *mef*A and *erm*B genes ($n=13$, 4.3%). Proportions of strains showing ML resistance were 85.9% of isolates from children and 77.9% of the isolates from adults.

Sensitivity and specificity of real-time PCR

As shown in Table 2, sensitivity and specificity for all strains were compared between conventional and real-time

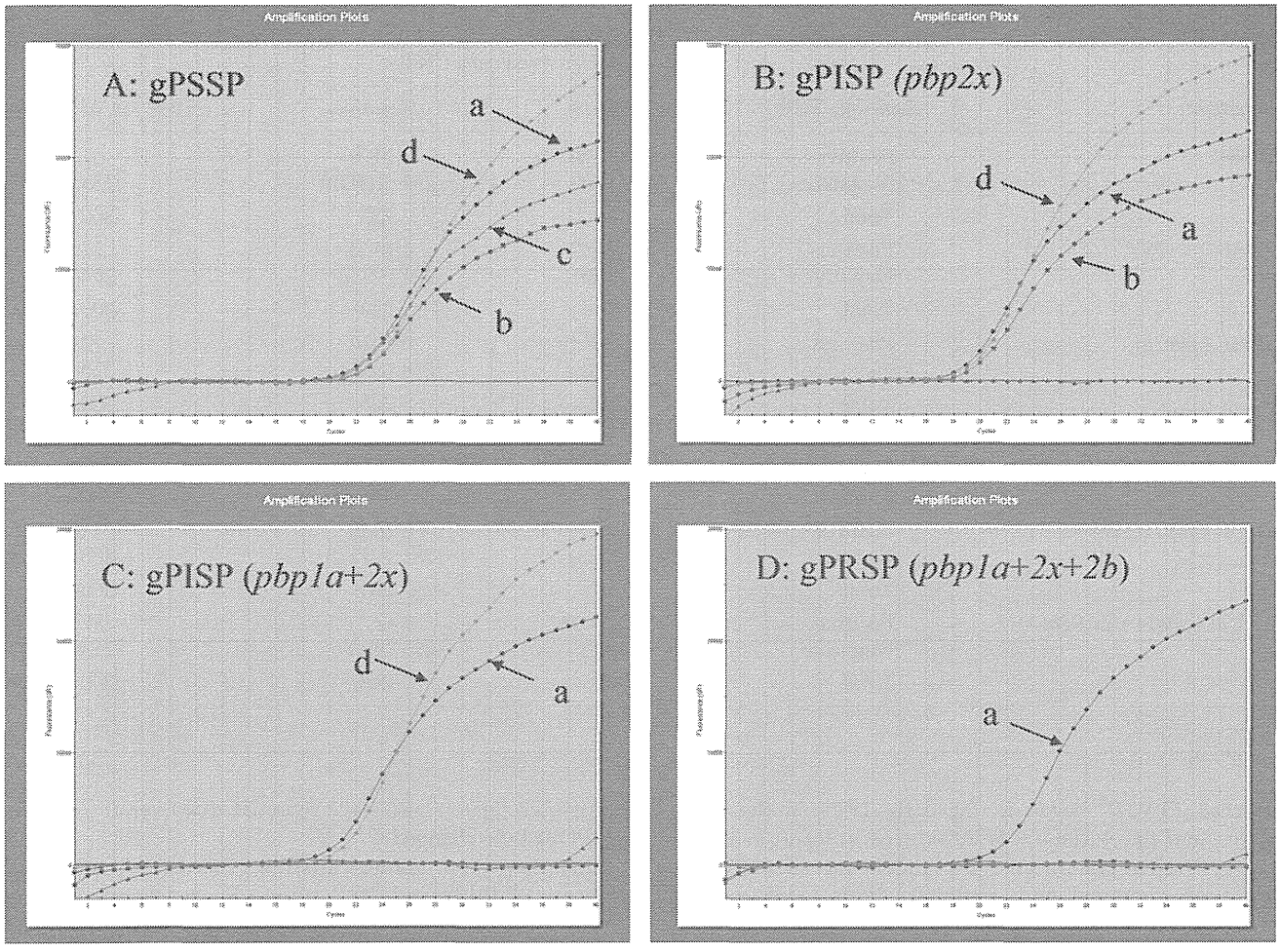

FIG. 1. Four genotypic resistance patterns from a computer display connected to the real-time PCR instrument, seen just after the PCR reaction was completed. (**A**) Genotypic penicillin-susceptible *Streptococcus pneumoniae* (gPSSP) by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes. (**B**) Genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*), with only the *pbp2x* gene not amplified. (**C**) gPISP (*pbp1a+pbp2x*), with *pbp1a* and *pbp2x* genes not amplified. (**D**) Genotypic penicillin-resistant *S. pneumoniae* (gPRSP) (*pbp1a, pbp2x,* and *pbp2b*), with 3 *pbp* genes not amplified.

PCR. The sensitivity and specificity for the *mefA* gene and the *ermB* gene were calculated to be 100%.

Table 3 shows detailed information for the nine strains (3.0%) showing a discrepancy between real-time PCR and conventional PCR. In these strains, DNA amplification for the *pbp1a* or *pbp2x* gene corresponding to the susceptible strain occurred weakly in conventional PCR but not at all in real-time PCR. According to susceptibility testing for AMP, and CTX, results of real-time PCR proved more accurate than those of conventional PCR. Overall, our new real-time PCR method showed to have excellent sensitivities and specificities compared with those of conventional PCR.

Relationships between PBP gene alterations and MIC of β-lactam agents

Figure 2 shows relationships between MICs of five β-lactam agents and results of real-time PCR for *pbp1a, pbp2x,* and *pbp2b* genes in the tested strains. MICs of PEN, MEM, and PAM were affected by *pbp2b* alterations rather than those in *pbp2x*. On the other hand, the MIC of CTX was 4–8 times lower than that of PEN due to *pbp2x* alterations. Notably, 90% of MICs in each genotype resistance class were distributed essentially within three serial dilution concentrations (for instance, gPRSP in PEN, from 0.5 to 2 mg/L) for almost all antibiotics. However, eight gPSSP strains with a CTX MIC ranging from 0.125 to 0.25 mg/L possessed substitutions of Thr550Ala adjacent to a KSG motif in PBP2X that could not be detect with the real-time PCR constructed in this study.

Estimated MIC_{50} values and corresponding ranges for 90% of β-lactam antibiotics among six PBP genotypic categories are listed in Table 4. On the basis of these data, MIC estimation for parenteral β-lactam antibiotics associated with clinical efficacy could be made with high probability.

Discussion

The ultimate global public health goal in the 21st century is to develop and disseminate vaccination to prevent infectious diseases caused by various viruses and bacteria more effectively. For immunity against pneumococcal infections, development of 23-valent pneumococcal polysaccharide