Incidence of Influenza after Vaccination in Southeast Osaka, Japan

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Abstract: *Objective*: The aim of this retrospective study was to evaluate the effect of influenza vaccine on the prevention of influenza in patients with lung diseases who live in southeast Osaka, Japan.

Method: We vaccinated 963 outpatients from November 2012 through December 2012 and followed the incidence of flu. In addition, from clinical records, we analyzed the age-ranked incidence of influenza A and B from April 2007 to March 2013.

Results: In the 2012–2013 flu season, Kaplan-Meier analysis showed that influenza A developed in 10 and influenza B developed in 0 of 963 patients with lung diseases. The incidence analysis showed that in the 2012–2013 season, vaccination against influenza A was not effective, whereas vaccination was effective against influenza B. According to the analysis, between 2007 and 2013, influenza B incidence in the local area was curbed, especially in the elderly, most of whom were vaccinated.

Conclusion: Annual flu vaccination effectively protects against at least influenza B in a local area in Japan. Additional studies are needed to evaluate the effectiveness of influenza B vaccination.

Keywords: Influenza A, Influenza B, Japan, vaccine.

1. INTRODUCTION

Influenza is a serious disease that can lead to hospitalization and sometimes even death. Every flu season is different, and influenza infection can affect people differently. Even healthy people can get very sick from the flu and spread it to others.

The influenza vaccination is an annual vaccination using a vaccine specific for a given year to protect against the highly variable influenza virus. [1] Each seasonal influenza vaccine in Japan contains antigens representing three (trivalent vaccine) influenza virus strains: one influenza type A subtype H1N1 virus strain, one influenza A subtype H3N2 virus strain, and either one or two influenza type B virus strains. [2] Influenza vaccines are administered as an injection in Japan.

Various public health organizations, including the World Health Organization, have recommended that annual influenza vaccination be routinely administered to patients at risk of developing influenza-related complications and to those individuals who live with or care for high-risk individuals, including the elderly (the United Kingdom recommendation is to vaccinate those 65 years of age or older), patients with chronic lung diseases (e.g., asthma or chronic obstructive pulmonary disease [COPD]), patients with chronic heart diseases (e.g., congenital heart disease, chronic heart failure, or ischemic heart disease), patients with chronic liver diseases (including cirrhosis), patients with chronic renal diseases (e.g., the nephrotic syndrome), patients who are immunosuppressed (those with HIV or who are receiving drugs to suppress the immune system such as chemotherapy and long-term steroids) and their household contacts, and people who live together in large numbers in an environment where influenza can spread rapidly, such as prisons, nursing homes, schools, and dormitories.

The effects of flu vaccination are controversial. Jefferson *et al.* [3] concluded that influenza vaccines are efficacious in preventing cases of influenza in children older than 2 years of age, but little evidence is available for children younger than 2 years of age. After a systematic review, Thomas *et al.* [4] concluded that vaccinating nursing home workers had no effect on confirmed influenza cases among the elderly residents. In another study, Thomas et al. [5] also concluded that vaccinating healthcare workers provided no benefit against the specific outcomes of laboratory-proven influenza or its complications (lower respiratory tract infection, or hospitalization or death due to lower respiratory tract illness).

Therefore, we retrospectively investigated the effect of flu vaccination on protection against the flu in patients with lung diseases who lived in the southeastern part of Osaka prefecture, Japan.

2. METHODS

We vaccinated 963 outpatients with lung diseases or gynecological disorders as well as pregnant women

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E-ISSN: 2310-9386/14

from November through December 2012 and retrospectively followed the incidence of laboratory-proven flu to the end of March 2013 from the clinical records database. The vaccination was an intradermal vaccination, standard in Japan, using the FLUBIC HA Syringe® (BIKEN, Japan). The observation period was from the start of the vaccination to 22 April 2013, and the endpoint was the development of influenza. The log-rank test was used to compare the survival times between the influenza A and B groups (Figure **1**). The diagnosis of flu was confirmed by the detection of influenza antigen using ESPLINE® Influenza A & B-N (FUJIREBIO Inc., Japan), which uses immunochromatography technology based on the principle of enzyme immunoassay. Both, the influenza A virus antigen and influenza B virus antigen, can be detected by a single test from nasal swab fluid, pharyngeal swab fluid, and nasal aspirate fluid. ESPLINE® Influenza A & B-N is a cassette-style reagent that is used in a simple procedure without any special instruments. The patients in our study included 398 men and 565 women with a mean age of 58 (range, 2–96) years. Because the sample size seemed to be small, to minimize bias, we chose a local area that is suitable for this type of study because it had a low population inflow and outflow, and because almost all patients with lung disease could be analyzed. Thus, we concluded that the sample size was sufficient for the study. In addition, we analyzed the age-ranked incidence of influenza A and B from April 2007 to March 2013. Because this was a retrospective study, ethics committee approval was not required.

3. RESULTS

Figure **2** shows the influenza incidence in the 2011–2012 (Figure **2A**) and 2012–2013 (Figure **2B**) flu seasons. All patients with flu-like symptoms at our medical center were thoroughly diagnosed using the influenza antigen detection kit. In the 2011–2012 flu season, 142 patients were influenza A-positive, 49 were influenza B-positive, and 685 were negative for both antigens. In the 2012–2013 flu season, 105 were influenza A-positive, 18 were influenza B-positive, and 497 were negative for both antigens.

The age distribution of the patients administered influenza vaccine in the area is shown in Figures **3** and **5A**. The age distribution of the patients administered influenza vaccination was bimodal, with peak ages at 40–49 and 70–79 years (Figure **5A**). We also show the age distribution according to disease classification, with the peak age range from 80–89 years in the patients with COPD, from 70–79 years in the patients with bronchial asthma, and from 40–49 years in the patients with gynecologic disorders or pregnant women (Figure **3**).

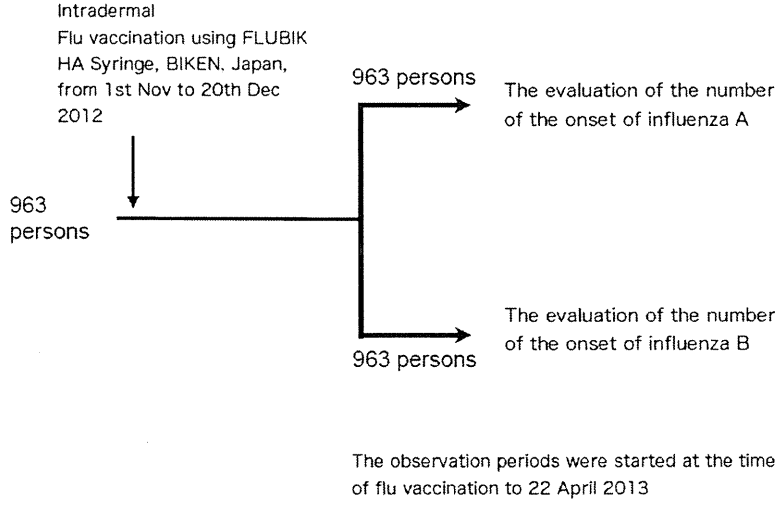

Figure 1: Flow diagram for the 963 outpatients administered influenza vaccination during the 2012–2013 flu season in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. The FLUBIC HA Syringe® in the 2012–2013 flu season contained California/7/2009(H1N1)pdm09 and Victoria/361/2011(H3N2) as protection against influenza A, and Wisconsin/1/2010 as protection against influenza B. We vaccinated 963 outpatients with lung diseases or gynecological disorders as well as pregnant women from 1 November to 20 December 2012 and retrospectively followed the incidence of laboratory-proven flu to the end of March 2013 from the clinical records database. The observation period extended from the date of vaccination to 22 April 2013. The endpoint was the development of influenza. The log-rank test was used to compare the survival times between influenza A and B groups.

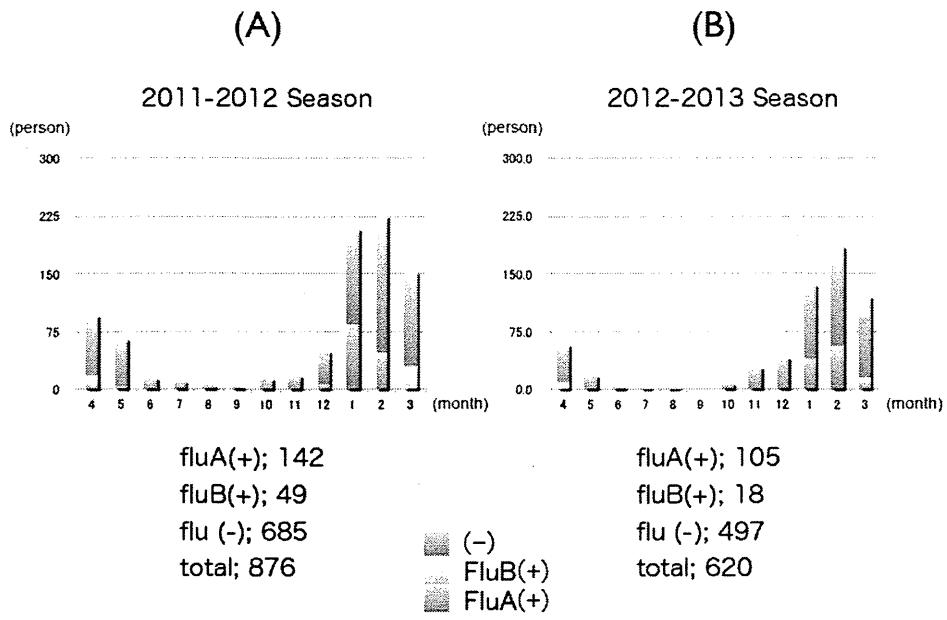

Figure 2: The age distribution of the patients with influenza A, influenza B, and influenza-like symptoms with no influenza antigen detected. Influenza antigens were detected using ESPLINE® Influenza A & B-N (FUJIREBIO Inc, Japan) in the 2011–2012 flu season (**A**) and in the 2012–2013 season (**B**) in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. The number of the patients having influenza symptoms such as fever >38°C, headache, muscle soreness, and coughing and/or dyspnea but without influenza antigens proven by ESPLINE® Influenza A & B-N is indicated as flu (-). The number of the patients with influenza A antigens proven by ESPLINE® Influenza A & B-N is indicated as fluA(+), and the number of patients with influenza B antigens proven by ESPLINE® Influenza A & B-N is indicated as fluB(+).

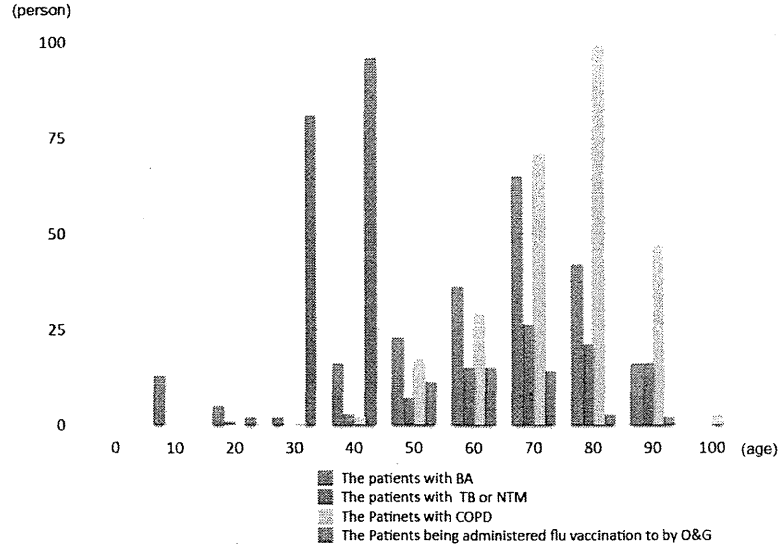

Figure 3: The age distribution of outpatients administered influenza vaccination using FLUBIC HA Syringe® (BIKEN, Japan) during the 2012–2013 flu season in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. Depicted are the age distributions of patients administered flu vaccination according to disease classification. The peak age range was from 80–89 years in the patients with chronic obstructive pulmonary disease (COPD), from 70–79 years in the patients with bronchial asthma (BA), and from 40–49 in patients with gynecologic disorders or pregnant women. Abbreviations: TB, active tuberculosis or complications after tuberculosis; NTM, nontuberculous mycobacterial; O&G, obstetricians and gynecologists.

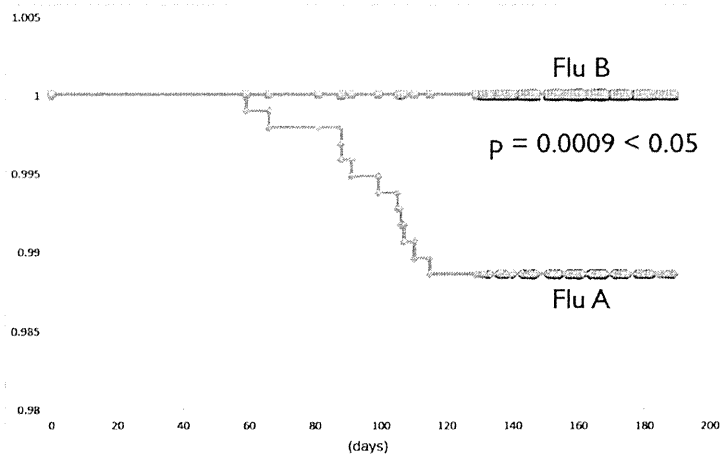

Figure 4: Kaplan-Meier analysis of influenza in patients with lung disease being administered influenza vaccination using FLUBIC HA Syringe® (BIKEN, Japan) during the 2012–2013 season in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. Influenza A developed in 10 and influenza B developed in 0 of 963 patients within 60 to 120 days after their flu shot. The log-rank test showed that the influenza A and B incidences were statistically different (P = 0.0009).

In the 2012–2013 flu season, Kaplan-Meier analysis showed that influenza A developed in 10 and influenza B developed in 0 of 963 patients within 60–120 days after their flu shot (Figure **4**). The log-rank test showed that the incidences of influenza A and B were statistically different (P = 0.0009).

The age distribution of the patients with influenza A is shown in Figure **5B**. There was no suppression of

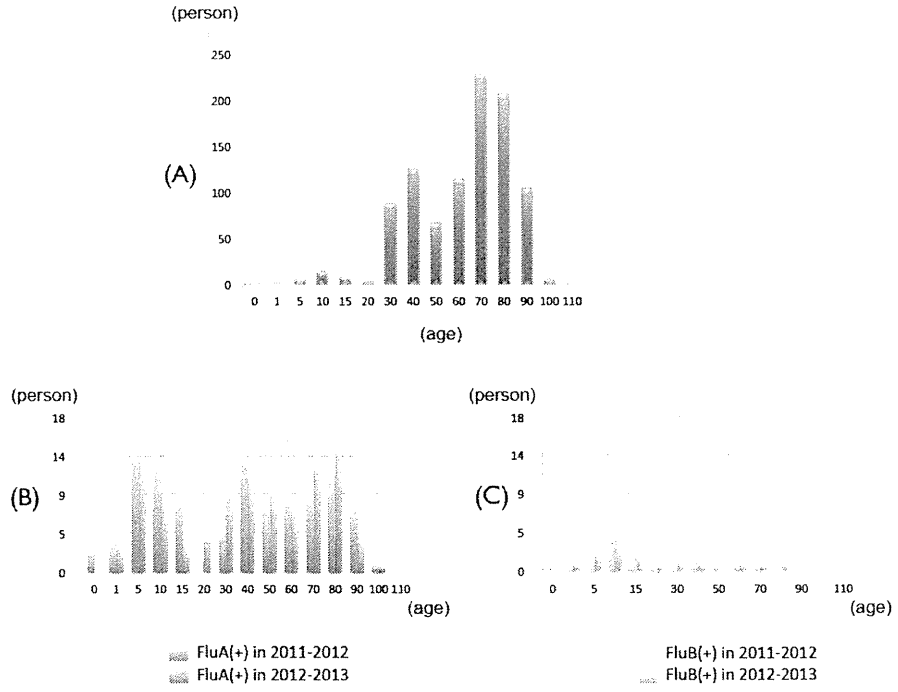

Figure 5: Age distribution of the patients administered influenza vaccination (**A**) and the distribution of the patients with laboratory-proven influenza A in the 2011–2012 and 2012–2013 flu seasons (**B**) and influenza B in the 2011–2012 and 2012–2013 flu seasons (**C**) in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. The patients with influenza shown in **B** and **C** included those who were not vaccinated. There was no suppression of influenza A incidence in patients 60 years of age or older (**B**), although most were vaccinated (**A**). In contrast, influenza B incidence was suppressed in patients 60 years of age and older (**C**).

influenza A incidence in patients 60 years of age or older, although most were vaccinated (Figure **5A**). In contrast, influenza B incidence was suppressed in patients 60 years of age or older (Figure **5C**).

According to the analysis, between 2007 and 2013 in the local area (Figure **6B**), influenza B incidence was curbed, especially in those 60 years of age or older, most of whom were vaccinated, but influenza A incidence in these patients, especially in the 2011–2012 and 2012–2013 flu seasons, was not curbed. The influenza A incidence in patients 60 years of age and older in the 2009–2010 flu season, when the H1N1 2009 pandemic virus prevailed, was curbed, with a distribution pattern similar to that of influenza B incidence.

4. DISCUSSION

Various public health organizations, including the World Health Organization, have recommended that annual influenza vaccination be routinely administered to patients at risk of developing complications from influenza and to those who live with or care for high-risk individuals. However, the effects of flu vaccination are controversial.

The Osaka Prefectural Medical Center for Respiratory and Allergic Diseases (the OPMCRAD) is a chest hospital located in southeast Osaka, Japan. Most of the patients with lung diseases who are 60 years of age or older are annually vaccinated against influenza. Because these patients were mainly located in the southeastern parts of Osaka, where population flow is

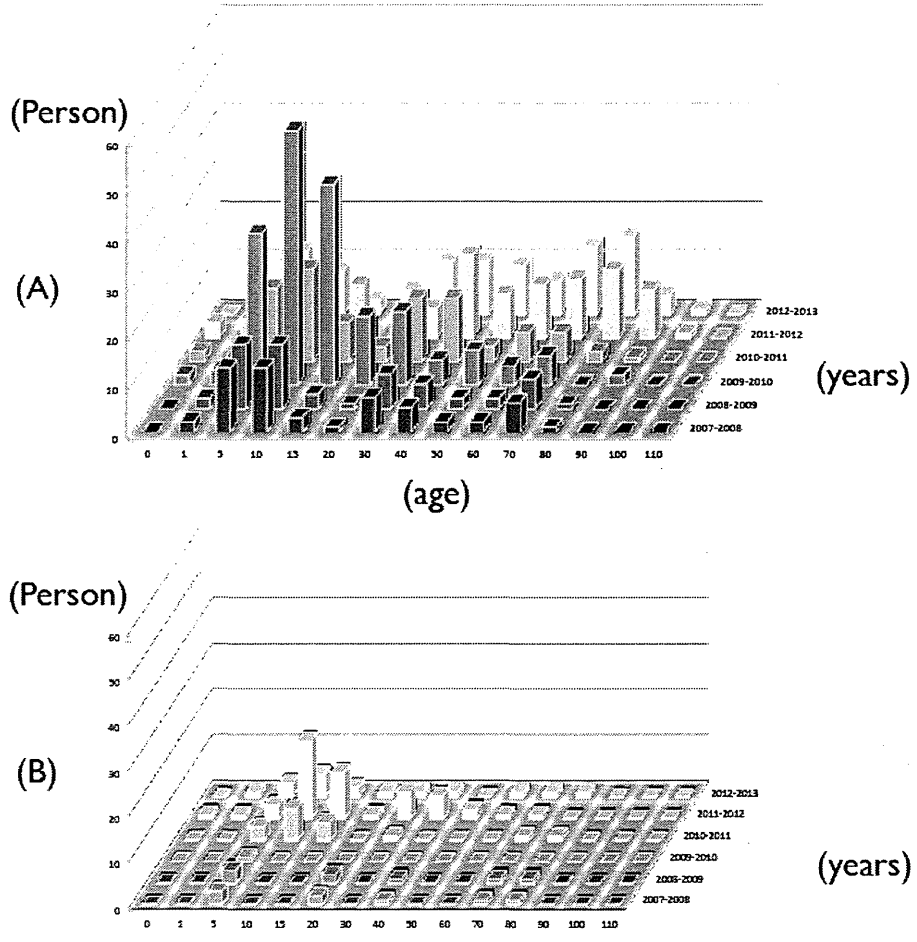

Figure 6: The numbers of influenza cases diagnosed by the detection of influenza antigen using ESPLINE® Influenza A & B-N (FUJIREBIO Inc, Japan) from 2007–2013 in the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, southeast Osaka, Japan. Between 2007 and 2013, the influenza A incidence (**A**) in patients 60 years of age and older, especially in the 2011–2012 and 2012–2013 flu seasons, was not curbed, although most of the patients in this age group were vaccinated. However, the influenza B incidence (**B**) was curbed, especially in patients 60 years of age or older.

small, when they developed flu-like illness, most of them visited the medical center. We counted the number of patients with influenza who were administered a flu shot in the OPMCRAD: 620 outpatient visits for flu symptoms with 497 laboratory-proven flu negative, 105 with influenza A, and 18 with influenza B.

Our data show that there was no influenza B in the vaccinated patients. One possible explanation of this could be a low influenza B prevalence in Japan during the 2012–2013 flu season. But, according to the data in the Japan Physicians Association Influenza Manual 2013–2014, [6] the influenza B prevalence was one-fourth of that of influenza A in each age group from 30–80 years. The total number of influenza B cases during the 2012–2013 flu season in the OPMCRAD was 18. The peak age distribution of influenza B was 10–14 years of age (6 cases), and the number of the influenza B cases among 20- to 80-year-old patients was suppressed. In contrast, the age distribution of influenza A was higher than that of influenza B in each age group, but especially in 60 years or older. The age distribution patterns of influenza B were similar to that of influenza A in the 2009–2010 flu season, when the vaccine was made and administered to protect from A(H1N1)2009pdm influenza and when the vaccination may have been effective.

It is generally accepted that flu vaccination is less effective against influenza B than against influenza A, but our data show that the flu shot was effective in preventing influenza B. The age-distributed influenza B incidence among the patients 60 years of age or older during the 2012–2013 flu season in Japan, shown by the Japanese Physicians Association, was not curbed compared to that of our data. [6] Therefore, we attribute the suppression of the influenza B incidence in our patients 60 years of age or older to the vaccination.

A study on vaccine effectiveness (VE) from the Centers for Disease Control and Prevention (CDC) measured lower VE against influenza A among people 65 and older in 2012–2013 compared with other age groups. [7] However, VE against influenza B was similar to what was seen in other age groups, whereas VE against influenza A (H3N2) viruses in people 65 and older was significantly lower than in other age groups. One possible explanation for this is that some older people did not mount an effective immune response to the influenza A (H3N2) virus component of this season's flu vaccine; however, it is not possible to say this for certain.

Our data confirmed the CDC's data but showed that VE against influenza B was more effective among people 65 and older. [7]

FluMist is the first live attenuated influenza vaccine and also the first nasally administered vaccine to be marketed in the United States for the prevention of flu in individuals 2–49 years of age. However, the effect of the intranasal vaccination on an older population is not known. Intradermal vaccination is a very important approach that can produce mucosal immunity and protection in immunized individuals, but intranasal vaccination can also be effective. [8] Because FluMist is not currently available in Japan, the effects of FluMist on influenza prevalence in Japan will be investigated in the future when it becomes available.

This study had some limitations because the area investigated and the study population were small. However, we could show the tendency for the flu vaccination to be effective in preventing influenza B because the area investigated has a low population inflow and outflow. Further study is needed.

5. CONCLUSION

Although we could not show the efficacy of influenza A vaccination in the prevention of influenza A in patients 60 years and older with lung diseases during the 2011–2013 flu season in southeast Osaka, our data showed that annual influenza vaccinations were efficacious in preventing influenza B in the patients of this age group.

ACKNOWLEDGEMENT

This study was supported by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan (H24-Shinkou-Ippan-010).

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Received on 05-06-2014 Accepted on 10-06-2014 Published on 15-06-2014

DOI: http://dx.doi.org/10.14205/2310-9386.2014.02.01.3

Incidence and Number of Reported Deaths due to Tuberculosis during Treatment with Biologic Agents in Japan

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Biologic agents have revolutionized the treatment of rheumatoid arthritis and other rheumatic diseases. The anti-tumor necrosis factor (TNF) inhibitors adalimumab, infliximab, and etanercept were listed in the ten top best-selling medications in 2012 [1]. Biologic agents other than anti-TNF inhibitors, such as actemra and abatacept, are also available in Japan. More types of biologics are expected to be used in the future because of the efficacies of anti-TNF inhibitors. Unfortunately, use of anti-TNF inhibitors has been reported to be associated with the development of tuberculosis (TB) [2, 3]. However, treatment of latent TB infection using isoniazid has been reported to reduce the incidence of TB in Japan [4]. We evaluated the morbidity and mortality of TB patients treated with biologics in Japan using the Japanese Drug Event Report Database (JADER). According to the JADER, from 2004 to 2012, TB developed in 408 patients (including redundant data) and 13 patients died. These data suggest that considerable attention should be paid to preventing TB in patients treated with biologics (Table **1**).

Table 1: Incidence and Number of Deaths of Patients with Tuberculosis Treated with Biologics in Japan

Fiscal Year	IFX	ETN	ADA	TCZ	GLM	ABT	CZP
2004	23						
2005	29	9		1			
2006	16(1)	11					
2007	31	8					
2008	24	11(2)	5	3			
2009	29	12(1)	9(1)	5			
2010	27	13	16(1)	2			
2011	36(1)	9(2)	14	5	1	2(1)	
2012	23	15(1)	14(1)	1	3(1)	1	
Total	238(2)	88(6)	58(3)	17(0)	4(1)	3(1)	0(0)

The number of tuberculosis incidence in each fiscal year. ( ); death toll

IFN, infliximab; ETN, etanercept; ADA, adalimumab; TCZ, tocilizumab; GLM, golimumab; ABT, abatacept; CZP, certolizumab.

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Received on 01-12-2013 Accepted on 11-12-2013 Published on 15-06-2014

DOI: http://dx.doi.org/10.14205/2310-9386.2014.02.01.2

E-ISSN: 2310-9386/14 © 2014 Pharma Publisher

Future of pharmacogenetics-based therapy for tuberculosis

Personalized medicine uses technology to enable a level of personalization not previously practical. Currently, tuberculosis (TB) therapy is not personalized. Previous reports have shown that a genetic polymorphism of *NAT2* is associated with large interindividual and inter-racial differences in the toxicity and efficacy of isoniazid. Herein, we show the safety and efficacy of a pharmacogenetics-based standard TB therapy and also provide a schematic presentation that proposed therapeutic approaches for latent TB infection (LTBI) using *NAT2* genotyping. Our data show that the pharmacogenetics-based TB therapy is safer and more efficacious than the standard therapy. Therefore, the therapy using *NAT2* genotyping proposed for LTBI herein will be safer and more efficacious than the standard LTBI therapy. Introduction of this therapy with *NAT2* genotyping will be one of the cornerstones of personalized medicine.

KEYWORDS: drug-induced liver injury isoniazid latent tuberculosis infection *N*-acetyltransferase 2 NAT2 personalized medicine pharmacogenetics pulmonary tuberculosis

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In 2011, an estimated 8.7 million cases of tuberculosis (TB; range: 8.3–9.0 million) were reported globally, with 125 cases reported per 100,000 individuals, and approximately 1.4 million people (range: 1.3–1.6 million) died of the disease [1]. In The Stop TB Strategy, the WHO recommends a standard 6-month four-drug regimen as the first-line therapy [2–4]. However, in some cases, the specified number of doses cannot be administered within the targeted time period because of drug toxicity [2,5]. Furthermore, drug-induced liver injury (DILI) can cause morbidity and even mortality [6–8]. These adverse drug events confer substantial additional costs such as the costs associated with increased frequency of outpatient visits, laboratory tests and hospitalization in more serious cases. Second-line anti-TB medications could cause greater toxicity-related problems and are often less effective than first-line medications, and treatment could be prolonged in spite of attendant challenges to ensure compliance. As a result, the risks of treatment failure and relapse increase.

Pharmacogenetics (PGx)-based testing is anticipated to be important across all medical specialties, as a pillar of the personalized medicine movement [9]. PGx-based testing involves genetic testing to assess the risk of adverse response development in a patient or likelihood of patient response to a given drug, facilitating drug selection and dosing [10]. PGx-based testing is a relatively new field and we will discuss the impact of PGx-based therapy for TB, and treatment of latent TB infection (LTBI) prior to primary preventive care.

Liver injury & PGx in anti-TB drug treatment

In most cases, anti-TB drug-induced liver injury (ATLI) has been caused by isoniazid (INH), a key drug of standard therapy. Genetically polymorphic *N*-acetyltransferase 2 (NAT2) metabolizes INH [11–13]. The INH elimination rate is trimodally distributed in accordance with NAT2 metabolic activity [14,15]. The status of NAT2 activity is genetically controlled and depends on the number of active alleles (*NAT2*4*, **12* and **13*). Variant alleles (*NAT2*5*, **6*, **7*, **14* and **19*) produce impaired NAT2 enzyme with lower activity. INH is first metabolized into acetyl-INH via NAT2 in the liver, followed by hydrolysis to acetylhydrazine. Acetylhydrazine is then oxidized into hepatotoxic intermediates by CYP2E1 [16]. Impairment of NAT2 activity and enzyme induction by rifampicin (RMP) likely shift it from the major pathways to alternative pathways that form the toxic metabolite hydrazine (FIGURE 1) [17].

Previous reports have shown that a genetic polymorphism of *NAT2* is associated with large interindividual and inter-racial differences in the toxicity and efficacy of INH [15,18–23]. Patients with slow acetylators (i.e., without any active alleles) develop hepatotoxicity more often than do the patients with rapid acetylators (i.e., two active alleles) during treatment for TB with the

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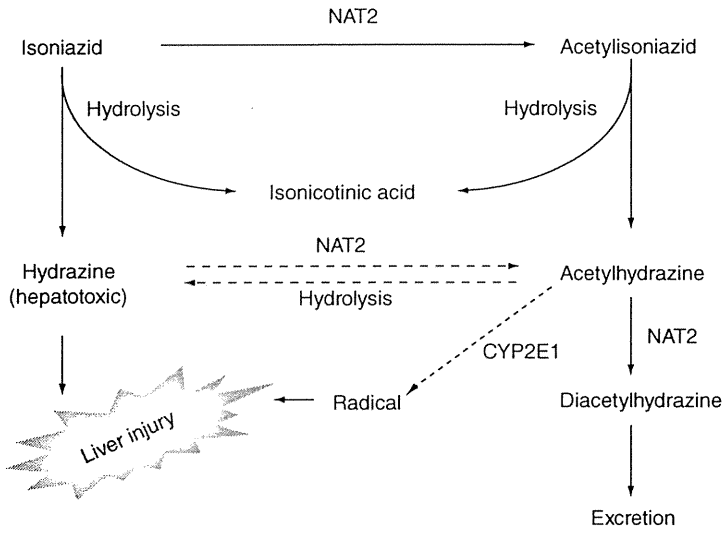

Figure 1. Metabolic pathways of isoniazid and incriminated metabolites that induce liver injury.

standard regimen [22,23]. It had been found that slow acetylators were present in almost 50% of Caucasians and only 10% of Asians. Meta-analyses have shown a significant association between NAT2 slow acetylators and a high risk of ATLI [24,25]. By contrast, rapid acetylators are shown to be prone to treatment failure with the standard regimen [15,18–20], probably owing to a low serum concentration of INH. These observations show that the current internationally recommended dosage of INH is too high for slow acetylators and insufficient for rapid acetylators. Thus, we believe that a pharmacogenetically stratified treatment would be useful to avoid INH-induced liver injury while improving the cure rate.

Meanwhile, CYP2E1 has been implicated since Mitchell *et al.* proposed a hypothesis that the enzyme generates radicals that attack hepatocytes [16]. It has been reported that *CYP2E1 c1/c1* homozygotes are prone to developing more severe hepatotoxicity than those with other *c1/c2* and *c2/c2* genotypes [26]. *CYP2E1 c2* is the allele named *CYP2E1*5* by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee [27]. A recent meta-analysis showed no statistically significant association between the *CYP2E1 c1/c1* genotype and ATLI (OR: 1.28; 95% CI: 0.97–1.69) [24]. Statistically significant risks associated with the genotype were found among Chinese populations, while no such significant associations were found among Japanese, Korean, Indian or Caucasian populations. Thus, the association between *CYP2E1* gene variants and ATLI is still controversial. Further PGx-based approaches are needed to provide new insights.

PGx-based standard TB therapy

A multicenter, parallel, prospective, randomized, open-label, blinded-end point (PROBE), controlled pharmacogenetic clinical trial was conducted in Japan to clarify whether *NAT2* genotype-oriented dosing of INH improves the efficacy and tolerability of the 6-month four-drug standard regimen for newly diagnosed pulmonary TB (NCT00298870) [28]. The eligible population was identified from a series of patients with newly diagnosed pulmonary TB who had normal liver and kidney functions, according to a blood biochemistry clinical examination. After *NAT2* genotyping, the patients were assigned to either conventional standard treatment (STD treatment: approximately 5 mg/kg of INH for all) or *NAT2* genotyped-guided treatment (PGx-based treatment: approximately 7.5 mg/kg for patients homozygous for *NAT2*4*: rapid acetylators; 5 mg/kg for patients heterozygous for *NAT2*4*: intermediate acetylators; 2.5 mg/kg for patients without *NAT2*4*: slow acetylators). The primary end points included incidences of INH-related DILI (INH-DILI) during the first 8 weeks of the therapy, and early treatment failure as indicated by a persistent positive sputum culture or no significant improvement in chest x-ray findings in the 8th week.

One hundred and seventy-two Japanese patients (slow acetylators: 9.3%, intermediate acetylators: 37.2% and rapid acetylators: 53.5%) were enrolled in the trial. After the allocation of the patients, *Mycobacterium avium* complex infections and resistance to INH were examined and used as additional exclusion criteria to ensure the efficacy of INH. Patients with complex infections or INH resistance were excluded from the final analysis. In this trial, all patients were treated as follows according to the Japan Anti-Tuberculosis Association (JATA) guidelines, with the exception of INH dosage, which was a 6-month regimen comprising INH, RMP, pyrazinamide and ethambutol/streptomycin for the first 2 months followed by RMP and INH for the remaining 4 months. This treatment schedule is in line with the current standard treatment recommended internationally for adult pulmonary TB [2].

Sample size to achieve a power of 0.8 with an α of 0.05 was calculated in the case of 22 slow acetylators and 156 rapid acetylators. A group sequential analysis showed that p-values regarding INH-DILI in slow acetylators were lower than the O'Brien–Fleming type stopping boundary determined using the Lan–DeMets approach. The trial was finalized early because of ethical considerations.

In the intention-to-treat analysis, INH-DILI developed in 78% of the slow acetylators in the STD treatment group, while none of the slow acetylators in the PGx-based treatment group developed INH-DILI or showed early treatment failure. Among the rapid acetylators, early treatment failure was observed with a significantly lower incidence rate in the PGx-based treatment group than in the STD treatment group (15 vs 38%). Thus, the *NAT2* genotype-guided regimen resulted in much lower incidences of unfavorable events (INH-DILI or early treatment failure) than did the conventional standard regimen. Further exploratory analyses were performed for 118 patients for whom positive sputum cultures were obtained at screening, who were infected with *M. tuberculosis* susceptible to all the first-line anti-TB drugs. In terms of primary outcomes, PGx-based treatment reduced INH-DILI while maintaining therapeutic efficacy in slow acetylators, and also reduced the incidence of persistent positive culture without increasing INH-DILI frequency in rapid acetylators (Figure 2). The incidence of unfavorable events in the combined population of rapid and slow acetylators was obviously higher in the STD treatment group than in the PGx-based treatment group. By contrast, PGx-guided dose stratification reduced unfavorable events to the degree observed in intermediate acetylators (Figure 3). With regard to other adverse events, there was no statistically significant difference in the incidence of these adverse events between the PGx-based treatment group and the STD treatment group.

In the randomized trial, *NAT2* genotype-guided dosing stratification of INH could improve treatment outcomes in patients with drug-susceptible TB. In slow acetylators, administration of half the conventional standard dose reduced the frequency of INH-DILI, but did not reduce therapeutic efficacy. In the case of rapid acetylators, administration of a dose 1.5-times the conventional standard dose of INH reduced the incidence of early treatment failure without increasing the incidence of INH-DILI. Thus, the results clearly demonstrate the potential of a *NAT2* genotype-guided dosing stratification of INH in chemotherapy for TB. Here, we propose that pharmacotherapy with INH against TB should be individualized by stratification based on *NAT2* genetic information in order to achieve the best outcome for each patient.

NAT2 genotyping

The frequency of the *NAT2* genotype differs across the world (Table 1); slow acetylator status occurs in more than 50% of Caucasians and in only 10% of Japanese. The effect of the *NAT2* genotype on the outcome of INH treatment cannot be ignored. As shown in previous studies, homozygous rapid acetylators show insufficient responses to the standard daily dose of INH (5 mg/kg, not more than 300 mg) [15,19] and slow acetylators are at risk for developing anti-TB DILI [22–25]. Based on pharmacogenetic dose-finding trials [29,30], the appropriate daily doses of INH estimated for slow acetylators, rapid acetylators and intermediate acetylators were 2.5 mg/kg, 7.5 mg/kg and 5 mg/kg, respectively. The validity of the estimated doses was verified in the randomized trial [28]. The next step is to begin applying *NAT2* genotype-guided dose stratification of INH in clinical settings. This new strategy against TB (Figure 4) will result in improved outcomes regardless of patient ethnicity.

PGx-based treatment requires genotyping in advance. Therefore, an inevitable issue is the risk associated with delay in treatment while waiting for genotyping. With regard to personalized medicine, the technology of genetic testing has improved considerably, especially when compared with traditional PCR-RFLP

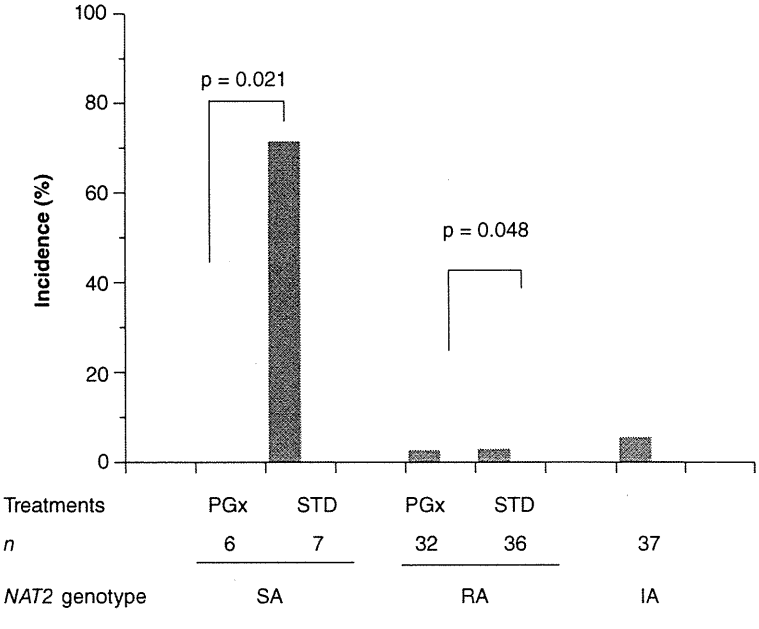

Figure 2. Incidence of isoniazid-induced liver injury and persistent positive culture among patients with drug-sensitive tuberculosis on sputum culture at screening. Purple columns: isoniazid-induced liver injury; blue columns: persistent positive culture.
IA: intermediate acetylator; PGx: Pharmacogenetics-based treatment; RA: Rapid acetylator; SA: Slow acetylator; STD: Conventional standard treatment.
Data taken from [28].

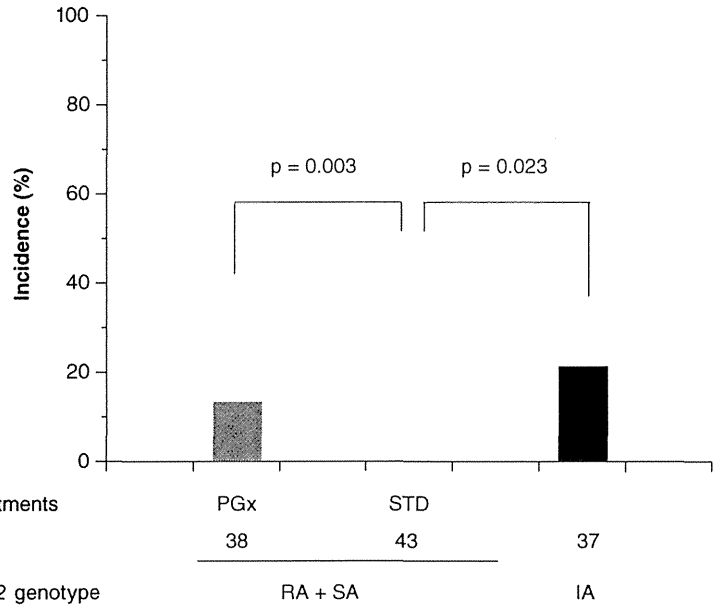

Figure 3. Incidence of combined unfavorable events among patients with drug-sensitive tuberculosis on sputum culture at screening.
IA: Intermediate acetylator; PGx: Pharmacogenetics-based treatment; RA: Rapid acetylator; SA: Slow acetylator; STD: Conventional standard treatment.
Data taken from [28].

methods. Commercially available methods are yet to be adopted in clinical practice. One of the rate-determining factors for the spread of PGx-based treatment in clinics is the development of handy, accurate and affordable genotyping equipment, such as equipment for multiplex SNP typing methods. Additionally, other factors such as cost of testing and insurance coverage are complicating issues.

The cost of *NAT2* genotyping for PGx-based treatment is difficult to estimate, because genotyping costs vary greatly depending on the number of samples, the testing method and facilities. Here is an example that might be a standard for genotyping-related pricing. The Ministry of Health, Labour and Welfare determined the testing cost of *UGT1A1*28* and **6* for irinotecan as 20,000 yen as a part of health insurance in Japan. In this case of *NAT2* genotyping for several SNPs, the fee for each patient was 5000 yen, but the price could be reduced to less than half after the customization of analysis procedure. On the other hand, treatment failures and adverse events during anti-TB treatment can lead to more clinical laboratory tests, more hospital stays and recovery delays; resulting in increasing costs.

LTBI

Immune-based tests, such as the tuberculin skin test or IFN-γ release assays (IGRA), have been used to diagnose LTBI. The risk of reactivation and development of active TB can be reduced by using therapy for patients with positive tests. The current standard LTBI therapy consists of INH, which can reduce the risk of active TB development by as much as 90% if taken daily for 9 months [32]. However, this long duration of therapy discourages patients, and the risk of serious adverse events (such as hepatotoxicity) discourages both providers and patients. As a result, the completion rate of INH therapy is less than 50% in many programs. Therefore, personalized, PGx-based therapy is needed to lower the incidence of adverse drug reactions.

Thus far, a series of pharmacogenetic observational studies have evaluated the potential association between *NAT2* gene polymorphisms and the risk of ATLI. It has been inferred that a *NAT2* genotype-based approach against both active and latent TB will be useful to improve the quality of anti-TB therapy. What is needed next is a study regarding *NAT2* genotype-based dose stratification of INH for LTBI therapy.

PGx-based LTBI therapy

Treatment of LTBI is important for controlling and eliminating TB [32], because it reduces the

Table 1. *NAT2* genotype frequencies in different regions of the world.

Region	Subjects (*n*)	NAT2 acetylator status based on genotyping		
		SA	*IA*	*RA*
Europe (Caucasian)	5382	0.58 (0.09)	0.34 (0.06)	0.08 (0.09)
Africa	1034	0.46 (0.19)	0.40 (0.14)	0.14 (0.14)
Asia	1790	0.45 (0.20)	0.37 (0.13)	0.18 (0.19)
Middle and South America	824	0.27 (0.18)	0.52 (0.15)	0.21 (0.16)
East Asia (Chinese, Korean and Japanese)	2062	0.14 (0.05)	0.46 (0.07)	0.40 (0.08)

SA = S/S, IA = R/S, RA = R/R. Slow alleles (S) = NAT2*5, *6, *7 *and* *14; *Rapid alleles (R)* = NAT2*4, *12 *and* *13.
Data are expressed as mean (standard deviation).
IA: Intermediate acetylator; RA: Rapid acetylator; SA: Slow acetylator.
Data taken from [31].

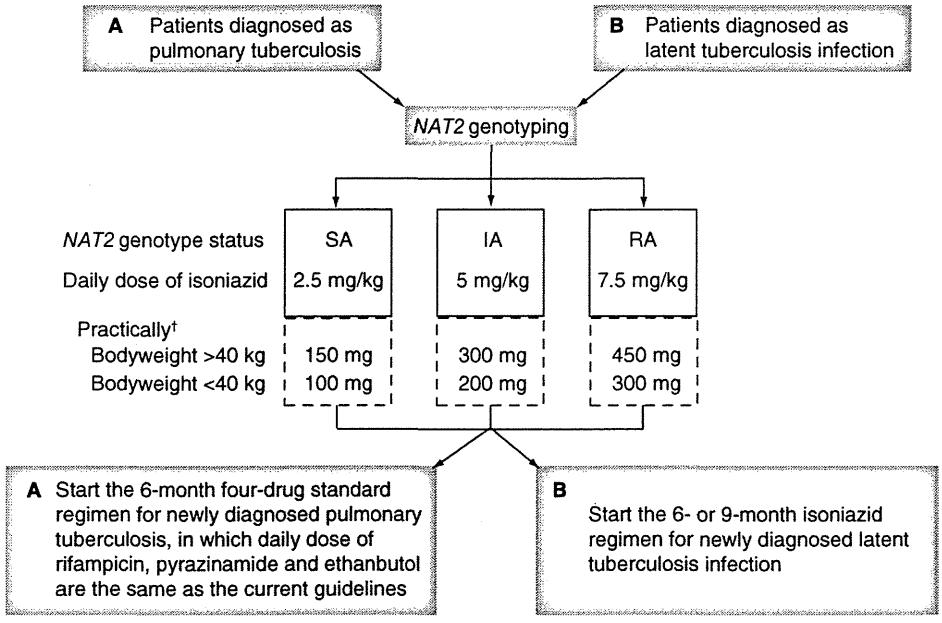

Figure 4. Proposal of pharmacogenetic antituberculosis therapy and pharmacogenetic latent tuberculosis infection therapy, where daily dose of isoniazid is optimized according to the individual's *NAT2* genotype status. (A) Pharmacogenetic antituberculosis therapy and **(B)** pharmacogenetic latent tuberculosis infection therapy, where daily dose of isoniazid is optimized according to the individual's *NAT2* genotype status.
SA has two slow alleles (two from a combination of *NAT2*5, *6, *7, *14* and **19*), IA has one slow allele and RA has no slow alleles.
†These doses are practical for using tablets of isoniazid.
IA: Intermediate acetylator; RA: Rapid acetylator; SA: Slow acetylator.

risk of TB in persons infected with *M. tuberculosis*. Some groups are at a high risk of developing TB once infected. Targeted testing programs have been designed to identify persons who are at a high risk for TB and who would benefit from treatment of LTBI. Targeted testing should be undertaken only if resources are identified and available to ensure full evaluation and treatment. There are two methods to detect *M. tuberculosis* infection in Japan and other industrialized countries, the Mantoux tuberculin skin test and IGRAs. In particular, the advent of IGRA caused an increase in the number of LTBI reported in Japan. Therefore, therapy for LTBI becomes increasingly important. LTBI treatment regimens are as follows: INH regimen, 12-dose (INH and rifapentine) regimen and RMP regimen.

There are two methods for treatment with INH: a 9-month regimen and a 6-month regimen. The 9-month regimen is used preferably because it is more efficacious than the 6-month regimen. On the other hand, treatment for LTBI for 6 months rather than 9 months is more cost effective and results in greater treatment adherence by patients; therefore, healthcare providers often use the 6-month regimen rather than the 9-month regimen. Every effort should and will be made to ensure that patients adhere to the LTBI treatment for at least 6 months. Therefore, it is important to ensure that LTBI treatment finishes with as few drug-induced adverse events as possible. PGx-based LTBI therapy will be increasingly important in this area.

Future perspective

Personalized medicine usually involves the use of technology to enable a level of personalization that was not previously practical. Personalized medicine involves the customization of healthcare, with medical decisions, practices and/or products being tailored for each patient. The use of genetic information has played a major role in some aspects of personalized medicine, and even the term itself was first coined in the context of genetics (although it has since broadened to encompass all sorts of personalization methods).

Personalized medicine has been applied in some fields (e.g., oncology) long before the term was coined. This is because oncology has a long history of classifying tumor stages and subtypes on the basis of anatomic and pathologic

findings, and this approach includes examination of genomic mutations in tumor specimens from individual patients (such as epidermal growth factor receptor mutations in lung cancer patients) to identify markers associated with prognosis and likely treatment responses. However, the principles underlying personalized medicine have still not been employed in TB therapy. Introducing therapy based on *NAT2* genotyping will be one of the cornerstones of personalized medicine [28].

Figure 4 shows our proposed schematic protocol of the *NAT2* genotyping-based therapeutic approach for TB and LTBI. Patients with LTBI were allocated into three categories (slow acetylator, intermediate acetylator and rapid acetylator) based on *NAT2* genotyping. INH was also adjusted by bodyweight, into high or low dose, in each genotype by group [28]. The duration of the therapy will be confirmed on the basis of the results of future clinical trials. On the basis of the findings of a research group on PGx-based therapy for TB [28], we believe that a similar therapy for LTBI will be safer and more efficacious than the standard therapy. Furthermore, to avoid side effects, patients who do not carry *NAT2*4*, slow acetylators, should be prescribed a lower dose of INH, and patients who carry only *NAT2*4*, rapid acetylators, should be given a higher dose to achieve the expected response. We expect PGx-based treatment for TB and LTBI to become the standard of care.

Financial & competing interests disclosure

This article was supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H24-Shinkou-Ippan-010). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Currently, personalized tuberculosis therapy is still not available.
- Previous reports have shown that a genetic polymorphism of *NAT2* is associated with large interindividual and inter-racial differences in the toxicity and efficacy of isoniazid.
- *NAT2* genotype-based tuberculosis therapy is obviously safer and more efficacious than the conventional standard therapy.
- Thus, the *NAT2* genotyping-based latent tuberculosis infection treatment proposed in this article will be safer and more efficacious than the standard latent tuberculosis infection therapy.
- Introduction of this therapy involving *NAT2* genotyping will be one of the cornerstones of personalized medicine.

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Journal of Antimicrobial Chemotherapy Advance Access published November 6, 2014

J Antimicrob Chemother
doi:10.1093/jac/dku443

Journal of Antimicrobial Chemotherapy

Molecular characterization of linezolid-resistant CoNS isolates in Japan

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Received 17 August 2014; returned 11 September 2014; revised 25 September 2014; accepted 13 October 2014

Objectives: Linezolid has been reported to remain active against 98% of staphylococci with resistance identified in 0.05% of *Staphylococcus aureus* and 1.4% of CoNS. The objective of this study was to characterize the linezolid-resistance mechanisms in the linezolid-resistant CoNS strains isolated in Japan.

Methods: *Staphylococcus capitis* strains exhibiting linezolid MICs >8 mg/L isolated from inpatients between 2012 and 2014 were screened for *cfr* and mutations in 23S rRNA, L3 and L4 by PCR/sequencing. Isolates were also examined for mutations in the *rlmN* gene.

Results: *S. capitis* had six 23S rRNA alleles. Five *S. capitis* isolates displayed linezolid MICs of 8, 16 and 32 mg/L. G2576U mutations were detected in three, four or five copies of 23S rRNA in all isolates. In two isolates exhibiting the highest linezolid MIC (32 mg/L) there was a large deletion in a single copy of 23S rRNA. Repeated 10 bp sequences were found in both 16S and 23S rRNAs, suggesting deletion by recombination between the repeats. One isolate had the mutation Ala-142→Thr in the ribosomal protein L3. All linezolid-resistant isolates also demonstrated mutations in the gene encoding RlmN methyltransferase, leading to Thr-62→Met and Gly-148→Ser.

Conclusions: Multiple mechanisms appeared to be responsible for the elevated linezolid resistance in *S. capitis* isolates: a G2576U mutation in different numbers of copies of 23S rRNA, loss of a single copy of 23S rRNA and a mutation in the ribosomal protein L3, suggesting the accumulation of independent mutational events.

Keywords: *S. capitis*, ribosomal mutations, *rlmN*

Introduction

Linezolid is the first agent of the oxazolidinone class to be introduced clinically and to remain consistently active against MDR Gram-positive bacteria, including MRSA and VRE.[1–3] Linezolid was first approved for clinical use in 2000 in the USA, Europe and other countries. Soon after its introduction to clinical use, in 2001, a linezolid-resistant MRSA recovered from a patient treated with this agent was reported in the USA.[4] Although multifocal outbreaks of linezolid-resistant staphylococci have been reported,[4–7] a recent article reviewing the literature concerning linezolid-resistant *Staphylococcus* infections mentioned that linezolid remains active against >98% of staphylococci, with resistance identified in 0.05% of *S. aureus*.[8] In Japan, linezolid was approved for treating MRSA infection in 2006, but has been closely restricted to ensure control of hospital-acquired infections. Despite this restricted use, 11 linezolid-resistant MRSA strains were identified in clinical isolates collected at six hospitals during 2006–08.[9] Furthermore, a case of linezolid-resistant MRSA isolated after long-term repeated use of linezolid at a hospital in Japan was reported in 2009.[10]

Methicillin-resistant CoNS and MRSA are major causes of both healthcare- and community-associated infections. Linezolid is not approved for treating patients with catheter-site or catheter-related bloodstream infections or infections where CoNS are commonly implicated, but the incidence of linezolid-resistant CoNS has increased. CoNS seem to develop linezolid resistance readily since linezolid-resistant strains have been identified in 1.4% of CoNS, but in only 0.05% of *Staphylococcus aureus*.[8] Linezolid-resistant CoNS reported in Europe comprised nine different species, among which 76.4% were *Staphylococcus epidermidis*, 9.1% were *Staphylococcus hominis* and 8.8% were *Staphylococcus haemolyticus*.

To our knowledge, linezolid-resistant CoNS have not been clinically isolated in Japan. During the interval May 2012 to April 2014

we isolated seven linezolid-resistant CoNS strains from inpatients at Chiba, Japan, including five strains of *Staphylococcus capitis* recovered from different patients. In the present study, the linezolid resistance mechanisms in these five clinical *S. capitis* isolates were characterized. Linezolid inhibits bacterial growth via protein synthesis inhibition by binding to the peptidyltransferase centre (PTC) in the 50S ribosomal subunit, thereby perturbing the correct positioning of aminoacyl-tRNA on the ribosome.[11–13] Modification of the ribosome at the PTC, commonly by mutation of domain V in 23S rRNA, has primarily been related to linezolid resistance in staphylococci.[14–18] However, a naturally occurring resistance gene *cfr*, encoding a Cfr methyltransferase that catalyses methylation at the C-8 position of A2503 in 23S rRNA, has more recently been reported in clinical isolates.[19–21] Although rRNA methylation is a common mechanism for acquiring antimicrobial resistance,[22] there is recent evidence that the endogenous RlmN that modifies the C-2 position of A2503 in 23S rRNA could be linked to linezolid susceptibility in *S. aureus*.[23,24] In the *S. capitis* isolates studied here, we detected G2576U mutations in three, four or five copies of 23S rRNA. In two isolates with the highest MIC of linezolid, a large deletion in a single copy of 23S rRNA was found in addition to the G2576U mutation. In all linezolid-resistant isolates, we demonstrated mutations in the gene encoding the RlmN methyltransferase. The present results provide insight into the multiple mechanisms of linezolid resistance in *S. capitis* strains clinically isolated in Japan.

Materials and methods

Bacterial strains

Five linezolid-resistant *S. capitis* strains (I-0553, I-0676, I-1184, I-2648 and I-0507) were characterized, with a linezolid-susceptible strain (I-0428) as control. All strains were isolated from inpatients during the period 2012–14 at a hospital in Chiba, Japan. The characteristics of the organisms and associated clinical data are listed in Table 1.

Antimicrobial susceptibility

Isolates were tested for antimicrobial susceptibility by the reference broth microdilution method using Mueller–Hinton broth in validated panels (DP32, Dryplate, Eiken Chemical), according to the CLSI method.[25] The MICs of linezolid were determined by serial 2-fold dilution using Mueller–Hinton agar plates. The MIC results were interpreted on the basis of published CLSI criteria.[26] Linezolid was purchased from Sigma-Aldrich Co.

PCR amplification and DNA sequencing

To test for mutations in individual copies of 23S rRNA by PCR amplification, the six specific primer sets described in Table S1 (available as Supplementary data at *JAC* Online) were used to amplify the copies of 23S rRNA from linezolid-resistant isolates individually. The resulting DNA fragments were sequenced using the same primers. For analysis of ribosomal protein genes by PCR amplification, followed by DNA sequencing, a specific primer set to amplify genes for L3 and L4, presented in Table S1, was used. The presence of *cfr* was investigated by PCR amplification using the forward primer 5′TGAAGTATAAAGCAGGTTGGGAGTCA and the reverse primer 5′ACCATATAATTGACCACAAGCAGC.

PFGE

PFGE was performed using the method described by Schnellmann *et al.*[27] with some modifications. Genomic DNA was prepared in agarose blocks and digested with SmaI (TaKaRa Bio, Japan). The DNA fragments were separated in a 1% agarose slab gel using a CHEF-Mapper system (Bio-Rad Laboratories Inc., CA, USA) for 27 h at 6 V/cm and 14°C, with a pulse angle of 120° and a ramped pulse time of 2.98–35.38 s. A CHEF DNA size standards lambda DNA ladder (Bio-Rad) was used as a reference marker.

Results

Characteristics of clinically isolated linezolid-resistant *S. capitis* strains

This study included five linezolid-resistant *S. capitis* strains recovered from different inpatients in a hospital at Chiba, Japan, during the interval May 2012 to April 2014. The susceptibility profiles of the linezolid-resistant *S. capitis* isolates I-0553, I-0676, I-1184, I-2648 and I-0507 are presented in Table 1. The MICs of linezolid ranged from 8 to 32 mg/L for these strains. These linezolid-resistant strains were resistant to oxacillin, imipenem, erythromycin, clindamycin, levofloxacin and minocycline. All isolates were susceptible to vancomycin, teicoplanin and arbekacin.

The clonal relatedness of the isolates was examined by PFGE of SmaI-digested genomic DNA (Figure 1). The five linezolid-resistant isolates were indistinguishable from each other, suggesting that they were derived from a similar clone. However, they were unrelated to the susceptible strain, I-0428, isolated in the same hospital. The linezolid resistance mechanisms in the five isolates were characterized.

Table 1. Demographic data and antimicrobial susceptibility profile of linezolid-resistant *S. capitis* isolates

			Resistance profile [MIC (mg/L)]									
Strain	Collection date	Clinical sample	LZD	VAN	TEC	OXA	IPM	ABK	ERY	CLI	LVX	MIN
I-0553	May 2012	vascular catheter	8	2	2	>4	>8	0.5	>4	>2	>4	8
I-0676	Jun 2012	digestive organ	16	2	2	>4	>8	0.5	>4	>2	>4	8
I-1184	Sep 2012	vascular catheter	16	2	1	>4	>8	0.5	>4	>2	>4	8
I-2648	Dec 2013	blood culture	32	2	2	>4	>8	0.5	>4	>2	>4	8
I-0507	Apr 2014	vascular catheter	32	2	4	>4	>8	0.5	>4	>2	>4	8
I-0428[a]	Apr 2014	blood culture	0.5	2	4	>4	4	1	>4	2	4	<2

LZD, linezolid; VAN, vancomycin; TEC, teicoplanin; OXA, oxacillin; IPM, imipenem; ABK, arbekacin; ERY, erythromycin; CLI, clindamycin; LVX, levofloxacin; MIN, minocycline.
[a]A linezolid-susceptible *S. capitis* strain, isolated in the same hospital, was included as the comparative control.

Analysis of individual copies of 23S rRNA and ribosomal protein genes in linezolid-resistant S. capitis isolates

Determination of the number of 23S rRNA alleles in S. capitis

Resistance to linezolid has been associated with mutations in the central loop of the domain V region of 23S rRNA. Prior to testing for 23S rRNA mutations in the linezolid-resistant isolates, the copy numbers of 23S rRNA genes in *S. capitis* were determined because strains of *Staphylococcus* are known to have five or six 23S rRNA operons.[28] For this purpose, the read sequences of the whole *S. capitis* SK14 genome deposited in NCBI by next-generation sequencing (ftp://ftp-trace.ncbi.nlm.nih.gov/sra/sra-instant/reads/ByRun/sra/SRR/SRR005/SRR005141/) were assembled on to the sequences of six copies of 23S rRNA in the genome of *S. epidermidis* RP62A (GenBank accession no. NC_002976) as a reference. The sequences used as reference included the region from 500 bp upstream to 1000 bp downstream of each gene *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE* and *rrlF*. GS Reference Mapper Software (Roche) was used for assembly. All six copies of 23S rRNA in *S. capitis* were successfully amplified by PCR using primer sets designed on the basis of the assembled data for *S. capitis* 23S rRNAs. The primer sequences are presented in Table S1. Nucleotide sequences for the six different copies were confirmed by sequencing the amplified products.

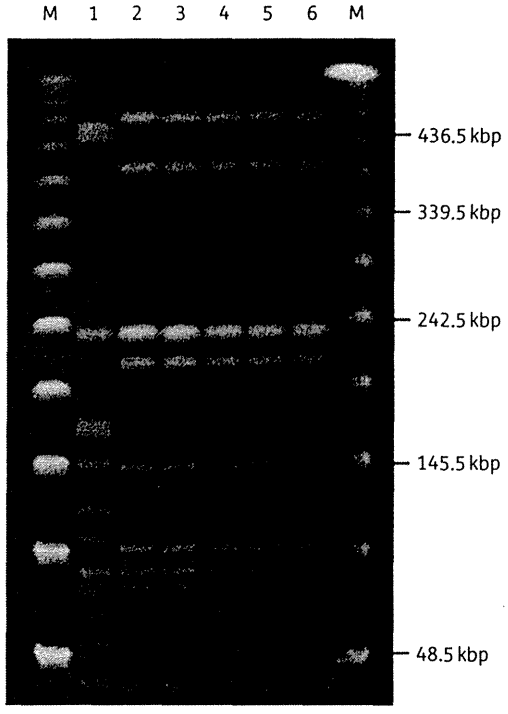

Figure 1. PFGE profiles of SmaI restriction digests of linezolid-resistant isolates of *S. capitis*. Lane M, CHEF DNA size standards lambda DNA ladder (Bio-Rad); lane 1, linezolid-susceptible strain I-0428; lanes 2–6, linezolid-resistant strains I-0553, I-0676, I-1184, I-2618 and I-0507, respectively.

Mutations in 23S rRNA alleles

The isolates were first tested for the most common mechanism, mutations in 23S rRNA. We designed PCR primers specific for each copy (Table S1), used them to amplify each copy individually and subsequently sequenced the copies. The G2576U mutation in domain V of 23S rRNA was identified in all isolates except the linezolid-susceptible one (Table 2). This mutation arose in different numbers of copies of the 23S rRNA gene: (i) three copies in strains I-0553 (MIC=8 mg/L) and I-0676 (MIC=16 mg/L); (ii) four copies in strain I-2648 (MIC=32 mg/L); and (iii) five copies in strains I-1184 (MIC=16 mg/L) and I-0507 (MIC=32 mg/L).

G2576U is the most frequently reported mutation in staphylococci,[4,11,12] although other mutations such as U2500A, U2504A and G2447U have also been identified in 23S rRNA in clinical isolates.[16–18] In the present isolates we found no mutations except G2576U, which has previously been associated with linezolid resistance.

Loss of a single copy of the 23S rRNA gene in isolates with high-level resistance to linezolid

Whereas PCR analysis detected six copies of 23S rRNA with the predicted size (4908 bp) in the linezolid-resistant strains I-0553,

Table 2. Characteristics of linezolid-resistant *S. capitis* isolates

	Mutations in 23S rRNA domain V allele sequence[a]						Mutations in ribosomal protein[a]			
Strain	*rrlA*	*rrlB*	*rrlC*	*rrlD*	*rrlE*	*rrlF*	L3	L4	*cfr*[b]	Mutation in RlmN
I-0553	—	—	—	G2576U	G2576U	G2576U	T83A	—	—	T62M/G148S
I-0676	—	—	—	G2576U	G2576U	G2576U	T83A	—	—	T62M/G148S
I-1184	G2576U	—	G2576U	G2576U	G2576U	G2576U	T83A/A142T	—	—	T62M/G148S
I-2648	deletion[c]	—	G2576U	G2576U	G2576U	G2576U	T83A	—	—	T62M/G148S
I-0507	deletion[c]	G2576U	G2576U	G2576U	G2576U	G2576U	T83A	—	—	T62M/G148S
I-0428[d]	—	—	—	—	—	—	T83A	—	—	—

[a]A long dash indicates no mutation.
[b]A long dash indicates that *cfr* was not detected.
[c]A 3018 bp deletion was found in the region covering the 3′-terminus of 16S rRNA and the 5′-terminus of 23S rRNA as represented in Figure 2.
[d]Linezolid-susceptible strain as the comparative control.

I-0676 and I-1184 and the linezolid-susceptible strain I-0428, analysis revealed that the *rrlA* gene was smaller (1885 bp) in one product, with five amplification products of the predicted size in strains I-2648 and I-0507 exhibiting increased resistance to linezolid (MIC = 32 mg/L). Figure S1 (available as Supplementary data at *JAC* Online) illustrates the agarose gel electrophoretic pattern of PCR-amplified products using the primer set for *rrlA*, indicating a DNA deletion had occurred in *rrlA* in the strains I-2648 and I-0507. Comparing the DNA sequence of the amplified products encapsulating *rrlA* with the corresponding region of *S. capitis* standard strain SK14 revealed that the 3018 bp sequence extending from the 3′-end of DNA for 16S rRNA to the 5′-end of DNA for 23S rRNA was lost in both I-2648 and I-0507 (Figure 2). In the standard strain SK14, we identified a 10 bp direct repeat sequence, GACGGGTGAG; one is in the region encoding 16S rRNA and the other is in that encoding 23S rRNA. The 3018 bp deletion could possibly have arisen by recombination between the two repeated sequences to generate a defective *rrlA* in I-2648 and I-0507.

Mutations in ribosomal protein genes

Linezolid resistance has also been associated with mutations in the ribosomal proteins L3 and L4, although both are located further from the bound drug.[29,30] All isolates in the present study had the mutation Thr-83→Ala in the L3 protein, which was not present in the control *S. capitis* SK14. Since Thr-83→Ala was also identified in the linezolid-susceptible isolate I-0428, it could not be responsible for linezolid resistance. An additional mutation, Ala-142→Thr, was found in the isolate I-1184 with an MIC of 16 mg/L. No mutation was identified in L4 in any isolate. Previous studies have reported mutations including Phe-147→Ile, Gly-139→Arg, Met-156→Thr and Ala-157→Arg in the L3 proteins of other species.[29,30] None of these mutations was found in any isolate in the present study.

Mutation of the endogenous RlmN methyltransferase in linezolid-resistant S. capitis isolates

In addition to the ribosomal gene mutations, acquisition of the 23S rRNA methyltransferase gene *cfr* is known to render staphylococci linezolid-resistant through modification of the C-8 position of A2503 in the PTC, thus preventing the drug from binding to the target site.[19–21] The *cfr* gene is found on plasmids and appears to be capable of horizontal transfer between staphylococcal species.[20,31–33] PCR demonstrated that no *cfr* is harboured by the linezolid-resistant *S. capitis* isolates characterized in the present study.

In addition to being the target for the Cfr methyltransferase, A2503 is also modified at the C-2 position by the endogenous RlmN methyltransferase, which is widespread and is found in the genomes of most bacteria.[34] A recently described *rlmN* mutation in an *S. aureus* isolate from a MRSA-infected patient treated with linezolid was thought to decrease susceptibility to the drug.[23] Furthermore, it was reported that a mutant lacking RlmN activity because of *rlmN* knockout outcompeted those with active RlmN under selective pressure from linezolid,[24] suggesting that loss of RlmN activity decreases susceptibility to linezolid. We therefore looked for mutations in *rlmN* on the genome of the linezolid-resistant *S. capitis*. The *rlmN* gene was individually amplified in all isolates using the primer set described in Table S1 and then subjected to DNA sequencing. Sequence analysis identified mutations in *rlmN* leading to Thr-62→Met and Gly-148→Ser in all linezolid-resistant isolates, but the gene was not altered in the linezolid-susceptible isolate I-0428 (Table 2).

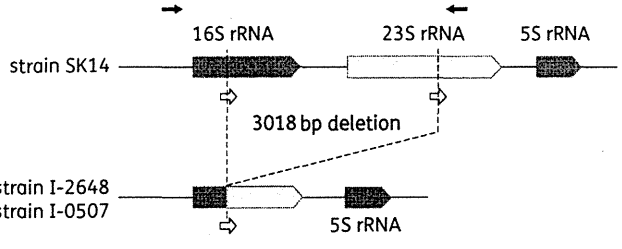

Figure 2. Schematic representation of a DNA fragment including *rrlA* in linezolid-resistant *S. capitis* isolates with a linezolid MIC of 32 mg/L compared with the corresponding region of the *S. capitis* standard strain SK14 from the database of the whole genome shotgun project (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=ACFR01). The sequences of PCR primer pair rrlA-F and domain V-R are given in Table S1. The black arrows indicate the regions hybridized by the primers. The open arrows indicate the GACGGGTGAG sequence repeat.

Discussion

S. capitis commonly inhabits human skin and mucosa, but is now recognized as an important opportunistic pathogen causing nosocomial bloodstream infections and indwelling catheter-related bacteraemia.[35] Recent studies have reported the emergence of methicillin-resistant *S. capitis* with reduced vancomycin susceptibility as an important cause of late-onset sepsis in neonatal intensive care units.[36,37] The incidence of linezolid resistance remains exceedingly low for staphylococci.[8] In this study, the resistance mechanisms in five *S. capitis* strains with various levels of linezolid resistance isolated from different inpatients between 2012 and 2014 were characterized.

Linezolid resistance has been associated with mutations in domain V of 23S rRNA and ribosomal proteins L3 and L4, and the acquisition of a transmissible Cfr ribosomal methyltransferase gene.[4,6,14–20,29,30] In the present linezolid-resistant isolates, we detected the mutation G2576U. This mutation was present in different numbers of copies of the 23S rRNA gene. Previously, *S. aureus* isolates were reported to demonstrate an increase in linezolid MIC as the number of mutant 23S rRNA genes increased.[15] In a series of *S. aureus* linezolid-resistant mutants, this gene dose effect has been described for mutations arising in the 23S rRNA after serial passages with linezolid, the number of mutated rRNA copies being directly related to the linezolid MIC.[38] Gene dosage effects have also been demonstrated in clinical *Enterococcus faecium* and *E. faecalis* isolates.[39] In contrast, the correlation is imperfect in the present series of *S. capitis* isolates, as seen by the discrepancy between I-0553 and I-0676, both of which had mutations in three operons, *rrlD*, *rrlE* and *rrlF*, yet had linezolid MICs of 8 and 16 mg/L, respectively. The difference in MIC could reflect other mutations, drug efflux or another unidentified mechanism. There was also a discrepancy between strains I-0676 and I-1184, both of which had the same linezolid MIC (16 mg/L), but had mutations in different numbers of 23S rRNA copies. On the other hand, there was a large deletion of 3018 bp extending

from the 3′ end of 16S rRNA to the 5′ end of 23S rRNA in the operon *rrlA* (Figure 2) in isolates I-2648 and I-0507, which exhibited the highest linezolid MIC (32 mg/L). The loss of a WT copy of the 23S rRNA gene, in the setting of existing mutant copies with G2576U, would increase the overall ratio of mutant to WT 23S rRNA copies. This would be expected to enhance the effect of the number of mutant copies present, resulting in an increased linezolid MIC.

The common mechanisms for linezolid resistance are the acquisition of a transmissible *cfr* methyltransferase gene and the ribosomal mutations. While mutational resistance to linezolid is troublesome in clinical practice, the acquisition of the *cfr* gene is more worrisome because of its rapid spread.[8,31–33] A very recent study reported the presence of *cfr* in almost half (48%) of linezolid-resistant *S. epidermidis* isolated in California between 2007 and 2012.[40] In contrast, no *cfr* was detected in any of the linezolid-resistant *S. capitis* included in the present study. On the other hand, the present results revealed that all *S. capitis* isolates except the linezolid-susceptible strain have uniform mutations, Thr-62→Met and Gly-148→Ser, in the endogenous RlmN methyltransferase that modifies the C-2 position of A2503, the C-8 position being methylated by the Cfr methyltransferase. In view of a previous report that an *rlmN* mutation in strains emerging from the parental MRSA in a patient after linezolid treatment increased the linezolid MIC from 0.74 mg/L (in the original MRSA) to 2 mg/L, it can be speculated that inactivation of the endogenous RlmN methyltransferase in *S. aureus* possibly increases linezolid resistance.[23] According to a recent report, an *S. aureus* strain lacking RlmN outcompeted those with active RlmN when both strains were co-cultivated in the presence of linezolid, suggesting that inactivation of RlmN in *S. aureus* increases linezolid resistance.[24] Although inactivation of *rlmN* seems to have little effect on linezolid resistance in staphylococci, the lack of RlmN-mediated modification might contribute to higher resistance in combination with other resistance factors, e.g. mutation of the 23S rRNA domain V.

We characterized the linezolid resistance in five linezolid-resistant isolates of *S. capitis* and revealed that multiple mechanisms are responsible: a G2576U mutation in different numbers of copies of the 23S rRNA gene, loss of a single copy of 23S rRNA and a mutation in the ribosomal protein L3. The results suggest the accumulation of independent mutational events rather than a single mutation in CoNS strains, presumably following exposure to linezolid, and subsequent spread throughout our health system. These findings underscore the need to develop strategies to prevent the emergence of linezolid resistance.

Funding

This work was supported by a grant (H24-Shinkou-Ippan-010) from the Ministry of Health, Labor and Welfare of Japan.

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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