化学物質の規制値は、乳児を含むヒトの健康への影響を保護するためにNOAELを不確実係数(UF)で割ることによって求められる。そこで、新生児と若齢ラット間のpNOAEL比を求めることによる新生児ラットの感受性を求めることは、リスクアセスメントにおける追加のUFを考える際に重要な資料となるかもしれない。

2-CP、4-CP、DMBP及びTHBの4つの化学物質のpNOAELは、新生児の方が1～5倍高い値であった。この結果はすでに報告した5つのフェノール系物質である4-nitro-、2,4-dinitro-、2,4,6-trinitro-、3-methyl- 及び3-aminophenolと同様であった(Koizumi et al. 2001, 2002, 2003; Takahashi et al. 2004)。これらの化学物質は多分直接的毒性発現物質であるため、新生児で第1相あるいは2相代謝酵素による解毒が未成熟であること(Rich & Boobis 1997; Gow et al. 2001)が新生児高感受性の主な原因であると推定される。HPMPに関しては、新生児のpNOAEL (100 mg/kg/day)は若齢 (40 mg/kg/day)の2.5倍であったが、若齢試験の200 mg/kgでの毒性は非常に軽微であることから、両者のpNOAELはむしろ同じレベルであると推定される。TTCに関しては、新生児は明らかに感受性が低い (pNOAEL比は0.2)。これに類似した結果はすでにbromoalkanesについての解析報告(Hirata-Koizumi et al. 2005)があり、これは新生児での毒性発現機構と代謝特性によって説明出来ると考えられる。このクラスの化学物質が毒性を発現するためには代謝を受ける必要があるが、若齢ラットでは成熟した代謝酵素による毒性中間体の生成が想定される(Onkenhout et al. 1986; Kennedy et al. 1993)。他の例として、acetaminophen、bromobenzeneあるいはcarbon tetrachlorideのような化学物質は成獣に肝毒性を発現する用量で新生児では肝障害を示さないことが示されている (Gergus & Klaassen 1998)

新生児と若齢ラットで同程度の毒性を発現する投与量であるpUETLの比は直接的毒性発現物質についてはpNOAELの場合と同じだった。しかし、HPMPに関しては、新生児のpNOAELが100 mg/kg/day、200 mg/kgで半分の動物が死亡したのに対して、若齢のpNOAELが40 mg/kg/day、1000 mg/kgで12匹中1匹しか死亡しなかったように、新生児の用量—反応性曲線は勾配が非常に急なため、pUETLの比較では新生児が明らかに高感受性であった。TTCに関しては、若齢ラットのpUETLは新生児とほとんど同じであった。このような結果は以前に解析を終了したbromoalkanesの場合にも見られた。低用量と高用量で異なった感受性を示す他の例としてはchlorpyrifosがある。成獣ラットの経口投与による最大耐用量は、17日齢のラットの5倍以上であるが(Moser & Padilla 1998)、低用量暴露の場合には感受性の違いは見られてない(Pope & Liu 1997)。このように、新生児の方が用量—反応性曲線が急勾配を示すいくつかの化学物質があるので、pUETL比もまた新生児感受性の第2指標として考慮すべきである。

E. 結論

2005年に公表されたWHOの水質ガイドラインの評価手法の各物質における特徴の傾向としては、LOAELの代わりにベンチマークドース(BMDL)を使用や動物実験からヒトへの外挿において、 PBPK手法を用いて標的臓器等における化学物質濃度の補正を行う。遺伝毒性発がん物質について、BMDLを出発点とした直線外挿を行っていることが挙げられた。

PBDEに関する毒性情報としては、限られた毒性データではあるが、多くは数mg/kg以上で、肝重量の増加とT4の減少、生殖発生影響が認められているが、胎生期および新生児期のより低用量での影響(精子数減少や行動異常など)が一部の同族体(BDE-47やBDE-99)で認められていることが明らかとなった。

6物質を用いた新生児ラットと若齢ラットの毒性指標に基づく感受性の比較検討では、pNOAEL及び

pUETL の比較に基づけば、殆どの場合に新生児ラットは若齢ラットより 2～5 倍高感受性であった。しかし、例外的に、TTC は若齢ラットの方が明らかに新生児ラットより感受性が高かった。

F. 研究発表

論文発表

Ema M, Kimura E, Matsumoto M, Hirose A, Kamata E. Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats. Reprod Toxicol (in press)

Ema M, Fukunishi K, Matsumoto M, Hirose A, Kamata E. Evaluation of Developmental Toxicity of Ultraviolet Absorber 2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole in Rat. Drug Chem Toxicol (in press)

高橋美加、松本真理子、川原和三、菅野誠一郎、菅谷芳雄、広瀬明彦、鎌田栄一、江馬 眞、OECD 化学物質対策の動向(第 8 報)－第 16 回 OECD 高生産量化学物質初期評価会議(2003 年パリ)、化学生物総合管理学会誌(印刷中)

松本真理子、高橋美加、平田睦子、広瀬明彦、鎌田栄一、長谷川隆一、江馬 眞、OECD 高生産量化学物質点検プログラム:第 18 回初期評価会議までの概要、化学生物総合管理学会誌(印刷中)

Hamamura M, Hirose A, Kamata E, Katoku K, Kuwasaki E, Oshikata T, Nakahara Y, Ema M, Hasegawa R. Semi-quantitative immunohistochemical analysis of male rat-specific alpha(2u)-globulin accumulation for chemical toxicity evaluation. J Toxicol Sci., 31: 35-47, 2006.

Takahashi M, Hirata-Koizumi M, Nishimura N, Ito Y, Sunaga M, Fujii S, Kamata E, Hasegawa R, Ema M. Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that in young rats. Congenit Anom Kyoto, 46: 26 -33, (2006)

Ema M, Hirose A. Reproductive and developmental toxicity of organotin compounds. Golub MS, Ed. Metals, Fertility, and Reproductive Toxicity, CRC Press, Boca Raton, 2006, pp. 23-64.

Hayashi M, Kamata E, Hirose A, Takahashi M, Morita T, Ema M. In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals. Mutat. Res., 588:129-135, 2005

Hirata-Koizumi, M., Hamamura M, Furukawa H, Fukuda N, Ito Y, Wako Y, Yamashita K, Takahashi M, Kamata E, Ema M, Hasegawa R. Elevated susceptibility of newborn as compared with young rats to 2-tert-butylphenol and 2,4-di-tert-butyl phenol toxicity. Congenit Anom Kyoto, 45, 146-153, 2005.

Hasegawa R, Hirata-Koizumi, M., Takahashi M, Kamata E, Ema M. Comparative susceptibility of newborn and young rats to six industrial chemicals. Congenit Anom Kyoto, 45, 137-145, 2005.

松本真理子、鈴木理子、川原和三、菅谷芳雄、江馬 眞、OECD 高生産量化学物質点検プログラム:第 20回初期評価会議概要, 化学生物総合管理学会誌 1, 445-453, 2005.

高橋美加、平田睦子、松本真理子、広瀬明彦、鎌田栄一、長谷川隆一、江馬 眞、 OECD 化学物質対策の動向(第 7 報)－第 15 回 OECD 高生産量化学物質初期評価会議(2002 年ボストン)、衛研報告、123, 46-52, 2005.

松本真理子、田中里依、川原和三、菅谷芳雄、江馬 眞、 OECD 高生産量化学物質点検プログラム:第 19 回初期評価会議概要, 化学生物総合管理学会誌 1, 280-288, 2005.

Hirata-Koizumi, M., Nishimura, N., Enami, T., Wada, H., Ogata, H., Yamamoto, Y., Ito, Y., Kamata, E., Ema, M. and Hasegawa, R. The susceptibility of new born rats to the hepatotoxicity of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, compared with young rats. J. Toxicol. Sci., 30, 29-42, 2005.

Ema M, Hara H, Matsumoto M, Hirose A, Kamata E. Evaluation of developmental toxicity of 1-butanol

given to rats in drinking water throughout pregnancy. Food Chem Toxicol, 43, 325-331, 2005.

高橋美加、平田睦子、松本真理子、広瀬明彦、鎌田栄一、長谷川隆一、江馬 眞、OECD 化学物質対策の動向(第 6 報)－第 14 回 OECD 高生産量化学物質初期評価会議(2002 年パリ)、化学生物総合管理学会誌、1, 46-55, 2005.

学会発表

江馬 眞、OECD 発生神経毒性試験ガイドラインについて、第 17 回神経行動毒性研究会、2005.

江馬 眞、藤井咲子、松本真理子、広瀬明彦、鎌田栄一、有機スズ化合物の生殖発生毒性:ジブチルスズのマウスにおける胚致死作用、第7回環境ホルモン学会研究会、2005.

Ema M, Kimura E, Hirose A, Kamata E. Reproductive and developmental toxicity screening test of 1,3-di-o-tolylguanidine in rats. EUROTOX 2005.

Hasegawa R, Hirata-Koizumi M, Takahashi M, Kamata E, Ema M. Susceptibility of new born rats to six chemicals, compared with young rats. EUROTOX 2005

江馬 眞、福西克弘、松本真理子、広瀬明彦、鎌田栄一、紫外線吸収剤 2-(3,5-di-tert-butyl-2-hydroxyphenyl)-5-chlorobenzotriazole のラットにおける発生毒性、第 32 回日本トキシコロジー学会学術年会、2005.

平田睦子、楠岡 修、西村信雄、和田 肇、緒方英博、福田苗美、伊藤義彦、鎌田栄一、江馬 眞、長谷川隆一、化学物質に対する新生児の感受性に関する研究:1,3-ジブロモプロパン及び 1,1,2,2-テトラブロモエタン、第 32 回日本トキシコロジー学会学術年会、2005.

江馬 眞、原 洋明、松本真理子、広瀬明彦、鎌田栄一、ブタノールのラットにおける発生毒性の検討、第 45 回日本先天異常学会学術集会、2005.

江馬 眞、OECD 神経発生毒性試験ガイドライン 426 (ドラフト)の進捗状況、第 45 回日本先天異常学会学術集会 BT シンポジウム、2005.

Hasegawa R, Hirata-Koizumi M, Takahashi M, Kamata E, Ema M . Susceptibility of newborn rats to six chemicals, compared to young rats. 42nd Congress of European Societies of Toxicology, Cracow, Poland. September, 2005.

Hirose A, Aisaki K, Hara H, Takahashi M, Igarashi K, Kanno J, Ema M. DNA Micro-Array Analysis of Gene Expressions in Mice Uterus Exposed with Dibutyltin Chloride during Implantation. The 25th International Symposium on Halogenated Environmental Organic Pollutants and POPs (DIOXIN 2005). Toronto, Canada, Aug, 2005.

Hirose A, Kanno J, Tokunaga H, Nakazawa K, Honma M, Inoue T. Initial investigation on the assessment of nanomaterial safety by the Japanese MHLW. 2nd International Symposium on Nanotechnology and Ocupational Health, Minneapolis, USA, Oct. 2005.

G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

(該当なし)

2. 実用新案登録

(該当なし)

3. その他

(該当なし)

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
遠藤卓郎 国包章一	2章　原虫	金子光美	水道の病原微生物対策	丸善	東京	2006	9-125
遠藤卓郎	6章　緊急時の対応	金子光美	水道の病原微生物対策	丸善	東京	2006	239-248

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
T. Nishimura, S. Iizuka, N. Kibune, M. Ando and Y. Magara.	Study of 1,4-dioxane intake in the total diet.	J. Health Sci.	51(4)	514-517	2005
F. Kura, J. Amemura-Maekawa, K. Yagia, T. Endo, M. Ikeno, H. Tsuji, M. Taguchi, K. Kobayashi, E. Ishii and H. Watanabe.	Outobreak of Legionnaires' disease on a cruise ship linked to spa-bath filter stones contaminated with *Legionella pneumophila* serogroup5.	Epidemiol Infect.	134	385-391	2006
Tadashi Itagaki, Shisuka Kinoshita, Mikiko Aoki, Naoyuki Itoh, Hideharu Saeki, Naoto Sato, Junya Uetsuki, Shinji Izumiyama, Kenji Yagita, Takuro Endo.	Genotyping of *Giardia intestinalis* from domestic and wild animals in Japan using glutamete dehydrogenase gene sequencing.	Veterinary Parasitology.	133	283-287	2005
遠藤卓郎、黒木俊郎	クリプトスポリジウム・ジアルジア感染症　ー話題の疾患と治療	感染・炎症・免疫	35(3)	77-79	2005
遠藤卓郎、八木田健司、泉山信司	いま、知っておきたい話題　クリプトスポリジウム症	日本醫事新報	4236	33-36	2005
黒木俊郎、泉山信司、遠藤卓郎	［話題の感染症］　クリプトスポリジウムの最近の知見.	モダンメディア	51(4)	75-80	2005
遠藤卓郎、八木田健司、泉山信司	＜特集　ヒトと動物の新興感染症＞　クリプトスポリジウム症	Medical Science Digest	31(1)	27-30	2005
T. Izumi, Y. Itoh, K. Yagita, T. Endo, T. Ohyama.	Brackish Water Benthic Shellfish (*Corbicula Japonica*) as a Biological Indicator for *Cryptosporidium parvum* Oocysts in Ricer Water.	Bull. Environ. Contam. Toxicol.	72	29-37	2004
遠藤卓郎、泉山信司	「病原微生物対策への理解に向けて」Safe Drinking-Water:for the Contorol of Microbial Hazards.	用水と排水	46(7)	43-49	2004

遠藤卓郎、黒木俊郎、泉山信司	＜話題の感染症＞ジアルジア症	モダンメディア	50(4)	73-77	2004
遠藤卓郎、黒木俊郎、泉山信司	レジオネラ症 Update －宿主アメーバからみたレジオネラの水系汚染対策	臨床と微生物	32(4)	383-388	2005
Ayano, E., Okada, Y., Sakamoto, C., Kanazawa, H., Okano, T., Ando, M. and Nishimura T.	Analysis of herbicides in water using temperature-responsive chromatography and an aqueous mobile phase	*J. Chromatogr. A,*	1069	281-285	2005
Tahara, M., Kubota, R., Nakazawa, H., Tokunaga, H. and Nishimura T.	Use of cholinesterase activity as an indicator for the effects of combinations of organophosphorus pesticides in water from environmental sources	*Water Res.*	39(20)	5112-5118	2005
Matsui, Y., Inoue, T., Matsushita, T., Yamada, T., Yamamoto, M. and Sumigama, Y.	Effect of uncertainties of agricultural working schedule and monte-carlo evaluation of the model input in basin-scale runoff model analysis of herbicides	*Water Sci. & Tech.*	51 (3-4)	329-337	2005
新井崇子，鎌田素之，島﨑大，浅見真理，相澤貴子	農薬の分子構造別の塩素分解性に関する研究	*水環境学会誌*	28(7)	437-444	2005
Ema M, Kimura E, Matsumoto M, Hirose A, Kamata, E.	Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats	Reprod Toxicol (in press)			
Ema M, Fukunishi K, Matsumoto M, Hirose A, Kamata, E.	Evaluation of Developmental Toxicity of Ultraviolet Absorber 2-(3′,5′-Di-tert-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in Rat	Drug Chem Toxicol (in press)			
高橋美加、松本真理子、川原和三、菅野誠一郎、菅谷芳雄、広瀬明彦、鎌田栄一、江馬眞	OECD化学物質対策の動向（第8報）－第16回OECD高生産量化学物質初期評価会議（2003年パリ）	化学生物総合管理学会誌(印刷中)			
松本真理子、高橋美加、平田睦子、広瀬明彦、鎌田栄一、長谷川隆一、江馬　眞	OECD 高生産量化学物質点検プログラム：第18回初期評価会議までの概要	化学生物総合管理学会誌(印刷中)			

Hamamura M, Hirose A, Kamata E., Kuwasaki E., Oshikata A. , Nakahara Y. Ema M, Hasegawa R.	Semi-quantitative immunohistochemical analysis of male rat-specific alpha(2u)-globulin accumulation for chemical toxicity evaluation	J Toxicol Sci.	31	35-47	2006
Takahashi M, Hirata-Koizumi M, Nishimura N, Ito Y, Sunaga M, Fujii S, Kamata E, Hasegawa R, Ema M.	Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that in young rats.	Congenit Anom Kyoto	46	26-33	2005
Ema M, Hirose A.	Reproductive and developmental toxicity of organotin compounds. Golub MS, Ed. Metals, Fertility, and Reproductive Toxicity	CRC Press, Boca Raton		23-64	2006
Hayashi M, Kamata E, Hirose A, Takahashi M, Morita T, Ema M.	In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals	Mutat. Res.	588	129-135	2005
Hirata-Koizumi, M., Hamamura M, Furukawa H, Fukuda N, Ito Y, Wako Y, Yamashita K, Takahashi M, Kamata E, Ema M, Hasegawa R.	Elevated susceptibility of newborn as compared with young rats to 2-tert-butylphenol and 2,4-di-tert-butyl phenol toxicity	Congenit Anom Kyoto	45	146-153	2005
Hasegawa R, Hirata-Koizumi, M., Takahashi M, Kamata E, Ema M.	Comparative susceptibility of newborn and young rats to six industrial chemicals	Congenit Anom Kyoto,	45	137-145	2005
松本真理子、鈴木理子、川原和三、菅谷芳雄、江馬眞	OECD 高生産量化学物質点検プログラム：第２０回初期評価会議概要,	生物総合管理学会誌	1	445-453	2005
高橋美加、平田睦子、松本真理子、広瀬明彦、鎌田栄一、長谷川隆一、江馬　眞	OECD 化学物質対策の動向（第７報）－第15回 OECD 高生産量化学物質初期評価会議（2002年ボストン）	衛研報告	123	46-52	2005
松本真理子、田中里依、川原和三、菅谷芳雄、江馬眞	OECD 高生産量化学物質点検プログラム：第19回初期評価会議概要	化学生物総合管理学会誌	1	280-288	2005

Hirata-Koizumi, M., Nishimura, N., Enami, T., Wada, H., Ogata, H., Yamamoto, Y., Ito, Y., Kamata, E., Ema, M. and Hasegawa, R.	The susceptibility of new born rats to the hepatotoxicity of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, compared with young rats.	J. Toxicol. Sci.	30	29-42	2005
Ema M, Hara H, Matsumoto M, Hirose A, Kamata E.	Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy.	Food Chem Toxicol	43	325-331	2005
高橋美加、平田睦子、松本真理子、広瀬明彦、鎌田栄一、長谷川隆一、江馬　眞	OECD 化学物質対策の動向（第 6 報）－第 14 回 OECD 高生産量化学物質初期評価会議（2002 年パリ）	化学生物総合管理学会誌	1	46-55	2005

Study of 1,4-Dioxane Intake in the Total Diet

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1,4-Dioxane is a newly added compound to the water quality standards in Japan that were revised in 2003. In order to estimate the contribution of 1,4-dioxane in drinking water to the total exposure in humans, it is necessary to take into account the quantity of the compound in food. In an earlier study, we measured the intake of 1,4-dioxane in food based on the average consumption of food in the Kanto area.[1] The total daily intake of 1,4-dioxane was calculated to be 0.440 μg. In the present study, we investigated the intake of 1,4-dioxane from food by sampling meals from 3 days from 3 homes in 9 prefectures, respectively. 1,4-Dioxane was extracted from 20 g of homogenates of mixed meals using the steam distillation, concentrated by a solid phase cartridge and then measured using gas-chromatography/mass spectrometry. The detection limit of the analysis was 2 μg/kg. No 1,4-dioxane was detected in 26 samples, while 3 μg/kg was detected in one sample. In this sample case, the daily intake of the 1,4-dioxane was calculated as 4.5 μg that represented 0.56% of the total daily intake (TDI) (4.5 μg/{16 μg/kg body weight/day × 50 kg}).

Key words —— 1,4-dioxane, total diet, risk, total meal

INTRODUCTION

1,4-Dioxane has been classified as a carcinogenic compound by both the USA Environmental Protection Agency[2] and the International Agency for Research on Cancer (IARC).[3] Long-term oral administration of 1,4-dioxane has been shown to cause tumors in the liver and gallbladder in guinea pigs,[4] and in the nasal cavity and liver of rats.[5–8] Studies in mice using a two-stage carcinogenic test have demonstrated 1,4-dioxane also has promoter activity.[9]

In 2003, 1,4-dioxane was added to the revised water quality standards in Japan. The compound is used extensively as an industrial solvent and is also added as a stabilizer to chlorinated solvents.[10] 1,4-Dioxane escapes to the aquatic environment and after discharging into the atmosphere returns to the surface as rainwater. As a result of its low adsorption to soil, 1,4-dioxane then permeates into the groundwater causing long term water pollution. As a consequence, 1,4-dioxane has the potential to cause widespread contamination of the environment and it is therefore important when evaluating exposure to the compound that every potential route of contamination is taken into account. Although there are several reports of 1,4-dioxane being detected in the environment,[11–13] there have been few reports on the content of 1,4-dioxane in food. Levels of 1,4-dioxane between 0.2 and 1.5 mg/l were detected in tap water samples collected between 1995 and 1996 from six cities in Kanagawa prefecture, Japan,[14] this finding raises the possibility that food may also have become contaminated. As there have been few reports on the contents and intake of 1,4-dioxane in food, in order to safeguard human health in Japan it is important to determine the intake levels of 1,4-dioxane in food.

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In an earlier study we measured the intake of 1,4-dioxane in food based on the average intake of food in the Kanto area of Japan as reported by the Ministry of Health, Labor and Welfare.[1] The total daily intake of 1,4-dioxane was calculated to be 0.440 μg. An intake of this magnitude corresponded to 0.055% of the calculated total daily intake (TDI) of 16 μg/kg body weight/day. In this paper, we extended these investigations by measuring the intake of 1,4-dioxane from meals.

MATERIALS AND METHODS

Chemicals —— 1,4-Dioxane was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), 1,4-dioxane-d_8 from Sigma-Aldrich Co., Ltd. (Tokyo, Japan), dichloromethane from Kanto Chemical Industry Co., Ltd. (Tokyo, Japan), ethanol from Katayama Chemical Industry Co., Ltd. (Osaka, Japan), acetonitrile and acetone from Wako Pure Chemical Industry Co., Ltd. (Osaka, Japan), and the antifoaming agent silicon TAS730 from Toshiba Silicon Co., Ltd. (Tokyo, Japan). All solvents were of the highest reagent grade. Purified water was prepared using a Milli-Q water purification PSS20 system (Millipore Corp., Bedford, MA, U.S.A.).

Preparation of Standard Solutions —— Standard solutions of 1,4-dioxane and 1,4-dioxane-d_8 were prepared in dichloromethane from stock solutions of 1 mg/ml 1,4-dioxane and 10 mg/ml 1,4-dioxane-d_8 as described previously.[1]

Preparation of Food Samples —— Samples of meals from 3 days were collected daily from 3 homes in 9 prefectures. The samples of the three meals cooked each day were added to samples of between-meal snacks and drink, followed by mixing and homogenization. The homogenates were then stored in glass bottles with silicon seals and kept frozen at –20°C until analyzed.

Extraction of 1,4-Dioxane —— 1,4-Dioxane was extracted from 20 g of each mixed meal homogenate using steam distillation as described previously reported.[1] Briefly, the samples were added to 150 ml of purified water and 100 μl of 2 μg/ml 1,4-dioxane-d_8 solution and then extracted, followed by concentration using a solid phase cartridge.

GC/MS Analysis —— Gas-chromatography/mass spectrometry (GC/MS) analysis was carried out using an Agilent 6890/5973N instrument (Agilent Technologies Inc., Palo Alto, CA, U.S.A.) instrument with an SPB-624 capillary column (60 m × 0.25 mm i.d. × 1.4-μm film thickness) (Sigma-Aldrich Co., Ltd.), as reported previously.[1] In the selected ion monitoring (SIM) mode, the monitoring ions were 58 and 88 for 1,4-dioxane and 64 and 96 for 1,4-dioxane-d_8. A calibration curve was prepared from the ratio of the peak height of 1,4-dioxane and 1,4-dioxane-d_8.

RESULTS AND DISCUSSION

Detection Limit in Food Samples

The minimum detection level of 1,4-dioxane added as an internal standard was 0.04 μg/l (S/N = 10), while the minimum detection limit of 1,4-dioxane in the prepared mixed meal was 2 μg/kg calculated using the following formula: (0.04 μg/l × 1 ml)/ 20 g = 0.002 μg/g = 2 μg/kg. In this formula, 1 ml represents the final volume of the GC/MS analysis and 20 g represents the weight of the mixed meal homogenate.

Recovery Test of 1,4-Dioxane

The concentration of 1,4-dioxane in the purified water used in the analysis was less than 0.04 μg/ml. After the addition of 0.2 μg of 1,4-dioxane and 1 μg of 1,4-dioxane-d_8 to 4 g of the prepared food samples, the recovery rate of 1,4-dioxane was obtained using the method described in the MATERIALS AND METHODS. The recovery rate of 0.2 μg of 1,4-dioxane was between 99 and 111% in the 12 food groups.[1] These results indicate that extraction of the compound from any food was efficient and met the requirements for this study.

Content of 1,4-Dioxane in the Mixed Meal Samples

The extraction of 1,4-dioxane from each of the 20 g prepared mixed meal samples was carried out according to the method described in the MATERIALS AND METHODS. Table 1 shows the content of 1,4-dioxane in the 27 mixed meal samples. 1,4-Dioxane was not detected in 26 of these samples but was detected in the remaining sample of a meal which collected on the first day from home C in the Nagano prefecture. The content of 1,4-dioxane detected in this sample was 3 μg/kg. Based on data reported by the Ministry of Health, Labor and Welfare the total weight of meals consumed each day is approximately 1.5 kg. Therefore, in the case of the positive sample the daily intake of the 1,4-dioxane was calculated to be 4.5 μg.

Table 1. Content of 1,4-Dioxane in the Mixed Meal Samples

Sample Site	Home Name	Day	Content (μg/kg)	Sample Site	Home Name	Day	Content (μg/kg)
Hokkaido	A	1st day	ND	Nagano Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	3
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Miyagi Prefecture	A	1st day	ND	Hyogo Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Tokyo	A	1st day	ND	Kagawa Prefecture	A	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	B	1st day	ND		B	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
	C	1st day	ND		C	1st day	ND
		2nd day	ND			2nd day	ND
		3rd day	ND			3rd day	ND
Aichi Prefecture	A	1st day	ND				
		2nd day	ND				
		3rd day	ND				
	B	1st day	ND				
		2nd day	ND				
		3rd day	ND				
	C	1st day	ND				
		2nd day	ND				
		3rd day	ND				

There is evidence that long-term oral administration of 1,4-dioxane in rodents causes hepatic and nasal cavity tumors in rodents,[8–12] and accordingly the IARC has classified 1,4-dioxane as a group 2B carcinogen.[6] With regard to a cancer endpoints, a TDI of 16 μg of 1,4-dioxane/kg body weight/day has been calculated by applying an uncertainty factor of 1000 to the level of 16 μg/kg body weight/day at which no adverse effects were observed in a long-term study of drinking water in rats.[15,16] This uncertainty factor incorporates 100 for inter- and intraspecies variation and 10 for nongenotoxic carcinogenicity. An intake of 4.5 μg of 1,4-dioxane corresponded to 0.56% of the TDI (4.5 μg/{16 μg/kg body weight/day $\times$ 50 kg}). As this proportion was the highest value in this investigation, we consider all

other proportions would be equal to or less than 0.56%. We therefore conclude that the intake of 1,4-dioxane from food appears to be very low and that this value does not increase the risk of carcinogenicity.

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REFERENCES

1) Nishimura, T., Iizuka, S., Kibune, N. and Ando, M. (2004) Study of 1,4-dioxane intake in the total diet using the market-basket method. *J. Health Sci.*, **50**, 101–107.
2) US EPA (1993) *Integrated Risk Information System (IRIS) on 1,4-Dioxane*, Environmental Criteria and Assessment Office, Office of Research and Development (Eds.), Cincinnati, OH.
3) IARC (1999) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 71, Pt 2, Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide*, World Health Organization, Lyon, pp. 589–602.
4) Hoch-Ligeti, C. and Argus, M. F. (1970) *Effect of carcinogens on the lung of guinea pigs. Morphology of experimental respiratory carcinogenesis*, Publication No. CONF-700401 (Nettesheim, P., Hanna, M. G., Jr. and Deatherage, J. W., Jr., Eds.), Technical Information Service, U.S. Department of Commerce, Springfield, VA, pp. 267–279.
5) Argus, M. F., Arcos, J. C. and Hoch-Ligeti, C. (1965) Studies on the carcinogenic activity of protein-denaturing agents: hepatocarcinogenicity of dioxane. *J. Natl. Cancer Inst.*, **35**, 949–958.
6) Argus, M. F., Sohal, R. S., Bryant, G. M. and Hoch-Ligeti, C. (1973) Dose-response and ultrastructural alterations in dioxane carcinogenesis. Influence of methylchlolanthrene on acute toxicity. *Eur. J. Cancer*, **9**, 237–243.
7) Hoch-Ligeti, C., Argus, M. F. and Arcos, J. C. (1970) Induction of carcinomas in the nasal cavity of rats by dioxane. *Br. J. Cancer*, **24**, 165–167.
8) Kociba, R. J., McCollister, S. B., Park, C., Torkelson, T. R. and Gehring, P. J. (1974) 1,4-Dioxane 1. Results of a 2-year ingestion study in rats. *Toxicol. Appl. Pharmacol.*, **30**, 275–286.
9) King, M. E., Shefiner, A. M. and Bates, R. R. (1973) Carcinogenesis bioassay of chlorinated dibenzodioxins and related chemicals. *Environ. Health Prespect*, **5**, 163–170.
10) Budavari, S. (Ed.) (1996) *The Merk Index*, 12 th edn., Merk & Co., Inc., Whitehouse Station, NJ, p. 3358.
11) Abe, A. (1999) Distribution of 1,4-dioxane in relation to possible sources in the water environment. *Sci. Total Environ.*, **227**, 41–47.
12) Ministry of the Environment (Planning and Coordination Bureau S) (1998) Outline of 1998 study results on designated chemicals, pp. 26–37.
13) Magara, Y., Sudo, R., Nakamuro, K., Yuasa, A., Abe, A., Sato, C., Sekine, H., Oosawa, E., Terasawa, K., Kawachi, T., Shiozaki, J., Aizawa, T., Ando, M. and Nishimura, T. (1998) Comprehensive approaches to the management of surfactants and related chemicals in water sources and drinking water treatment processes. In *Environmental Research in Japan, 1998* (National Resarch Institutes of Government Ministries and Agencies, Ed.), No. 1, Ministry of the Environment (Japan), pp. 8.1–8.33.
14) Abe, A. (1997) Determination method for 1,4-dioxane in water samples by solid phase extraction-GC/MS. *J. Environ. Chem.*, **7**, 95–100 (in Japanese).
15) Kochiba, R. J., McCollister, S. B., Park, C., Torkelson, T. R. and Gehring, P. J. (1974) 1,4-Dioxane. I. Results of a 2-year ingestion study in rats. *Toxicol. Appl. Pharmacol.*, **60**, 287–300.
16) World Health Organization (Ed.) (2004) *WHO Guideline for Drinking-water Quality*, 3rd edn., World Health Organization.

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Outbreak of Legionnaires' disease on a cruise ship linked to spa-bath filter stones contaminated with *Legionella pneumophila* serogroup 5

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SUMMARY

In January 2003, two cases of Legionnaires' disease associated with a ship's cruise were registered in the database of National Epidemiological Surveillance of Infectious Diseases. A 70-year-old male heavy smoker with mild emphysema contracted the disease during a cruise. *Legionella pneumophila* serogroup (sg) 5 was isolated from the patient's sputum and the ship's indoor spa. The isolate from the spa matched the patient's isolate by genotyping performed by pulsed-field gel electrophoresis (PFGE). The second case was in a 73-year-old female. During epidemiological investigation, a third case of Legionnaire's disease in a 71-year-old male was subsequently diagnosed among passengers on the same ship on the following cruise. Environmental investigation revealed that porous natural stones (*Maifanshi*) in the filters of the spas had harboured *L. pneumophila*, a phenomenon which has not been reported except in Japan. This is the first documented evidence of *L. pneumophila* sg 5 infection on a ship and of porous stones as a source of *Legionella* infection.

INTRODUCTION

Legionnaires' disease is a severe form of atypical pneumonia often caused by *L. pneumophila*, which in the United States from 1980 to 1998 accounted for 91·4% of clinical isolates [1]. Of the *L. pneumophila* isolates whose serogroup (sg) was known, 85% were sg 1, and only 1·9% were sg 5. In Japan from 1999 to 2002, 91% of clinical isolates for Legionnaires' disease were *L. pneumophila*; and among known serogroups, 65% and 3·8% were *L. pneumophila* sg 1 and sg 5 respectively [2]. Similarly, most ship-associated cases have been ascribed to infection by *L. pneumophila* sg 1, and isolation of sg 5 has been rare [3], only one being previously reported, in a Pacific cruise out of Sydney [4]. A molecular link between the disease and a source of infection on a ship has been documented in only two clusters caused by *L. pneumophila* sg 1 [5, 6].

In this report, we describe an outbreak of Legionnaires' disease caused by *L. pneumophila* sg 5 which was molecular-epidemiologically linked to the spa baths during the two cruises on the same ship.

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MATERIALS AND METHODS

Description of the cases

Case 1 (the index case in this cluster)

A 70-year-old Japanese man had been a passenger on a cruise ship with his wife from 27 December 2002 to 5 January 2003. They had never stayed ashore overnight. He had used the men's indoor spa bath at least seven times during the cruise. He presented with dry cough and mild fever since 1 January and with diminished appetite and general fatigue since 6 January [7]. On 13 January, he was admitted to the emergency room with severe dyspnoea. *L. pneumophila* sg 5 was isolated from the patient's sputum collected on the day of admittance, and urinary antigen was detected by Binax NOW *Legionella* immunochromatographic test (Binax Inc., Portland, ME, USA) in a specimen collected on hospital day 3. He rapidly developed multiple organ failure accompanied by septicaemia. Although the patient was discharged from the intensive care unit on day 22 of his hospitalization, the disease resulted in chronic need for supplemental oxygen.

Case 2

A 73-year-old female passenger on the same cruise ship had complained of cough and fever since 3 January. On 14 January, she was hospitalized for dyspnoea and severe pneumonia [8]. Biotest EIA test (Biotest AG, Dreieich, Germany) and Binax NOW *Legionella* immunochromatographic test detected urinary antigen of 'serogroups 1 and/or 5'. Culture and PCR of a sputum specimen were negative. By microplate agglutination test, single-serum antibody titres against *L. pneumophila* sg 1 to sg 6 were also negative. In spite of macrolide and aminoglycoside antibiotic therapy and steroid pulse therapy, she developed a pulmonary thromboembolism but on 16 March 2003 was finally discharged from the hospital. She had used the women's indoor spa bath in the cruise. Her home bath was not a spa, and the bath water was emptied after use every day. She had used neither pool nor spa except for the women's indoor spa and possibly also the whirlpool spa on the ship. There was no fountain in the public gardens near her home. No legionella was isolated from the female patient's home bath.

Case 3

A 71-year-old male passenger on the subsequent cruise from 7 to 9 January 2003, had presented with cough since 11 January and with fever of 38–39 °C since 12 January. He consulted a physician and was diagnosed without roentgenography as having bronchitis [9]. He had used the men's indoor spa bath twice a day for 3 days, but neither a whirlpool spa nor a warm pool during the cruise. After the epidemiological investigation had begun, he was informed of the occurrence of ship-associated Legionnaires' disease and visited the local community hospital on 30 January. By Biotest EIA test, his urinary antigen of probable 'serogroup 5' was positive and he was diagnosed as having Legionnaires' disease and was hospitalized on the same day. Sputum culture and paired sera test were both negative. Pathological examination revealed bronchitis and interstitial pneumonia. He was discharged on 15 February. His home bath was not a spa and he had not travelled except for the cruise.

Infection of other passengers

The cruise ship was a 26500-ton Japanese passenger ship with capacity for 696 passengers and 204 crew members. The first cruise of the ship was in 1998. There were 238 passenger rooms on the ship, of which 40 rooms had baths and the others showers. On 28 January 2003, based on one confirmed case and a probable source of infection in the cruise ship's environment, 1821 of the 1833 passengers who had travelled in a total of five separate cruises on the same ship from 27 December 2002 to 19 January 2003 were informed of the occurrence of Legionnaires' disease associated with the ship. All were advised to consult a physician in case of illness. This resulted in the finding of case 3, and an additional 18 passengers with illness (two with pneumonia, and the other 16 with fever, cough, nausea, and/or diarrhoea) after the cruises. However, the two other pneumonia patients were diagnosed as not having Legionnaires' disease, and no further case was found. The numbers of passengers on the first and second cruises, where confirmed cases were found, were 459 and 514 respectively. About 200 passengers enjoyed the indoor spas every day and the male : female ratio of indoor spa users was 3 : 2. Users of the whirlpool spa on the deck were fewer than 10 a day.

Environmental sampling

On 23 December 2002, three water samples collected from spas and one cabin's bath on the ship were tested by a private inspection company. These specimens

were obtained before any remedial decontamination. Further water samples collected from the spas on 14 January 2003 were tested at a legally incorporated foundation. All environmental samples mentioned above had been voluntarily collected by the ship's company for *Legionella* tests before the confirmation of the first patient. On 28 January 2003, when the outbreak was first suspected, a public health centre in Hyogo prefecture, where the ship was docked, performed environmental investigations. Sterile swabs were dipped in sterile saline, rotated against a 10-cm^2 surface area, kept and transported in a tube containing 10 ml of saline. Three residual water samples and 12 swabs were collected by public health officers at different sites on the ship, especially the spas. Two samples of porous natural stones (*Maifanshi*) used as filter bed material in the indoor-spa filters were collected on the same day, immersed and transported in sterile water. These stones and their immersion water were tested separately. *Maifanshi* is also called China medical stone. It is found in Inner Mongolia and in Japan, and is a kind of intrusion of granodiorite porphyry composed of aluminosilicates [10]. In recent years, *Maifanshi* have been used in Japan for producing artificial mineral water and as a refrigerator odour killer.

Laboratory methods

Water samples and swab suspensions were concentrated by filtration (0·22 μm pore size). The stones were put into an airtight container and sonicated in saline at 130 W and 20 kHz for 24 s to collect residual biofilms from inside the stones. The turbid extract was concentrated by centrifugation. Diluted and undiluted specimens were plated on buffered charcoal yeast extract (BCYE) α-non-selective and WYO α-selective agars (Eiken Chemical, Tokyo, Japan) [11], with and without heat or acid treatment, following the usual procedures for the isolation of *Legionella* spp. Identification of *Legionella* isolates was performed using species-specific and serogroup-specific monovalent antisera (Denka Seiken, Tokyo, Japan). Some isolates were confirmed by PCR with specific primers for *Legionella* 5S rRNA DNA and the *L. pneumophila mip* gene [primers were prepared according to the sequences from the formerly commercially available EnviroAmp *Legionella* (PerkinElmer Cetus Corporation, Norwalk, CT, USA)]. All available *Legionella* strains isolated from samples were sent to the *Legionella* Reference Centre, Department of Bacteriology in the National Institute of Infectious Diseases in Japan. To show the link between clinical and environmental *L. pneumophila* sg 5 isolates, strains were analysed by use of PFGE of genomic DNA digested with *SfiI* restriction enzymes [12]. Unfortunately, the isolates from the samples tested at the private inspection company were discarded after testing, which made retests impossible.

To isolate amoebae from the filter stones, 5–6 stones of 1–2 cm in diameter (whole weight, 33–35 g) were washed by inverting the tube for 1 min in a 50-ml centrifuge tube with sterilized water added up to 50 ml. The washing solution was transferred to a new 50-ml centrifuge tube and the volume of the solution was adjusted to 50 ml by adding sterilized distilled water. Five millilitres of the washing solution was concentrated to 1 ml in a 15-ml centrifuge tube by centrifuging at 500 ***g*** for 5 min. The sediment was resuspended and inoculated onto a non-nutrient agar plate coated with heat-inactivated *Escherichia coli*. Test plates in a plastic bag were incubated at 30 °C and examined for 7 days for growth of amoebae [13].

Some *L. pneumophila* sg 5 strains previously isolated from different origins [Fig. (*b*)] were used for comparison of DNA fingerprints.

RESULTS

Environmental investigations

The circulating system in the men's indoor spa was identical to but independent of that in the women's indoor spa. Each bathtub contained 5·5 m^3 of water. The water in the tubs was changed twice a day. According to the test report of the inspection company on water samples collected on 23 December before the first cruise, water samples from the indoor men's spa and a whirlpool spa on the deck had yielded legionellae, while water from a royal cabin bath had not (Table); but that fact was unfortunately not reported to the ship's company until after the cruise. Because the indoor spa water showed high contamination by legionellae (1·5 $\times 10^4$ c.f.u./100 ml, a spa for males), these spas were disinfected by circulation of water with a high concentration of chlorine (5–7 mg/l) for 7 h [9], resulting in decreased *L. pneumophila* sg 5 c.f.u. in water samples collected from an indoor (male) spa and a whirlpool spa on 14 January. However, legionellae were still not eradicated from the spas, and subsequent samples exhibited a legionella concentration of 8·0 $\times$ 10 to 2·9 $\times 10^3$ c.f.u./100 ml (Table).

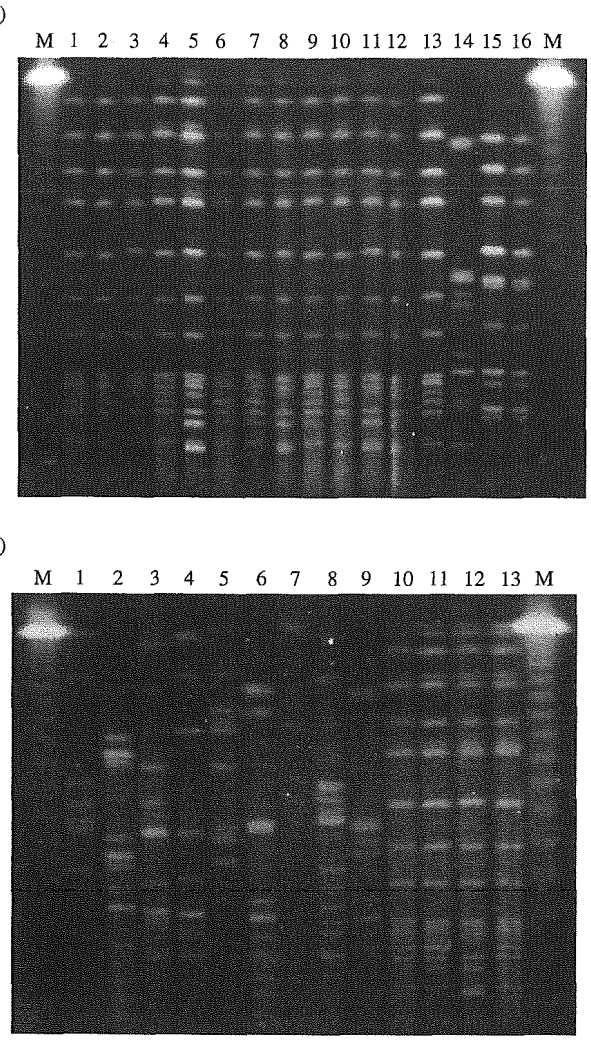

Fig. Macrorestriction analysis of chromosomal DNA derived from *Legionella pneumophila* sg 1 and sg 5 digested with *Sfi*I and separated by pulsed-field gel electrophoresis. (*a*) A comparison of the clinical isolates with the strains isolated from water samples and swabs at different sites of the ship. Lanes 1 and 2, sg 5 isolates from patient (case 1) sputum; lane 3, sg 5 isolate from men's spa water; lanes 4–8, sg 5 isolates from natural stones in men's spa filter; lanes 9–12, sg 5 isolates from natural stones in women's spa filter; lane 13, sg 5 isolate from a strainer of women's spa; lane 14, sg 5 isolate from a whirlpool spa; lane 15, sg 1 isolate from a swab at a strainer for women's spa; lane 16, sg 1 isolate from natural stones in women's spa filter. (*b*) *L. pneumophila* sg 5 strains. Lane 1, NIIB 412, Osaka LG02-11 from a spa bath; lane 2, ATCC 33216 Dallas 1E from a cooling tower; lane 3, NIIB 98 (EY 3420), a clinical isolate in Osaka [22]; lane 4, NIIB104 (EY 3427), a clinical isolate in Kurashiki; lane 5, ATCC 33737 U8W from shower head water; lane 6, NIIB 288 Ishioka 1-2-4 from a spa bath [18]; lane 7, NIIB 330 (ThaiNIH 7811) from a cooling tower; lane 8, NIIB 361 (ThaiNIH 10723) from a cooling tower; lane 9, corresponding to lane 14 of panel (*a*); lane 10, corresponding to lane 1 of panel (*a*); lane 11, corresponding to lane 4 of panel (*a*); lane 12, corresponding to lane 5 of panel (*a*); lane 13, corresponding to lane 6 of panel (*a*). Ms are DNA size markers, lambda ladders (Bio-Rad, Richmond, CA, USA), as indicated on the right and left sides of each electrophoregram.

After a passenger was diagnosed with Legionnaires' disease, administrative inspection of the ship was begun and environmental samples were collected from different sites at the spas and in the male patient's cabin on 28 January. Of the environmental water samples collected on 14 and 28 January, most were negative or soon became negative as a result of the crew's and others' cleaning efforts. The surfaces of the porous stones appeared clean, and no amoebae, often a reservoir of legionellae, were isolated from the stones. But some legionellae were detected in the water surrounding the stones during transit to the testing, and sonication dislodged large amounts of legionellae from inside the porous stones. As a result, it was found that some cleaning of the stones had been achieved, but not eradication of legionella. All 36 isolates from men's spa stones were *L. pneumophila* sg 5, 14 of 15 isolates from women's spa stones were *L. pneumophila* sg 5, with one isolate sg 1. All other isolates from the men's spa and women's spa were identified as *L. pneumophila* sg 5, and a mixture of *L. pneumophila* sg 1 and sg 5 respectively.

L. pneumophila subtyping

We obtained isolates from only case 1. Genomic fingerprints (PFGE) of *L. pneumophila* sg 5 isolates from the patient and various sites of the ship's spas show that the same fingerprint strains as the clinical isolates (lanes 1 and 2) were obtained [Fig. (*a*)] from stones in both spa filters for men (lane 4) and women (lane 10) and from the men's spa water (lane 3). Interestingly, genomic fingerprints of stone isolates revealed a small variation [lanes 4–12, Fig. (*a*)]. Some isolates showing one or two additive band(s) with one band's disappearance in PFGE patterns were observed, suggesting that the porous stones harboured a group of strains derived from a common ancestor. The fingerprints of a *L. pneumophila* sg 5 strain from the water of the whirlpool spa and of a *L. pneumophila* sg 1 strain from the women's spa water (lanes 14 and 15 respectively) were different from the fingerprint of the clinical isolates.

We examined whether the variation in PFGE patterns of *L. pneumophila* sg 5 strains is wide enough to discriminate between clinical and environmental isolates. As shown in Figure (*b*), each *L. pneumophila*

Table. *Isolation and characterization of clinical and environmental* Legionella *strains with the cruise ship*

Sampling site	Specimen (date of sampling)	*Legionella* (c.f.u./100 ml water) (c.f.u./100 g stone)	*Legionella* isolates serogroup	Genomic type of Lp5
Patient from cabin A	Sputum		Lp5	A
Indoor spa for males	Bath water (23 Dec.)	$1{\cdot}5 \times 10^4$	Lost	
	Bath water (14 Jan.)	$2{\cdot}9 \times 10^3$	Lp5	A
	Bath water (28 Jan.)	Not isolated		
	Strainer water (28 Jan.)	Not isolated		
	Natural stones (28 Jan.)	$3{\cdot}3 \times 10^2$	Lp5	A, A′, A″
Indoor spa for females	Bath water (14 Jan.)	$1{\cdot}0 \times 10^3$	Lp1	
	Bath water (28 Jan.)	Not isolated		
	Shower head incrustations (28 Jan.)	Not isolated		
	Natural stones (28 Jan.)	$7{\cdot}1 \times 10$	Lp1, Lp5	A, A′
	Strainer incrustations (28 Jan.)		Lp5	A
Whirlpool spa	Bath water (23 Dec.)	$1{\cdot}4 \times 10^2$	Lost	
	Bath water (14 Jan.)	$8{\cdot}0 \times 10$	Lp5	B
Cabin A (Case 1)	Shower head incrustations (28 Jan.)	Not isolated		
Cabin B (Case 2)	Shower head incrustations (28 Jan.)	Not isolated		
Bath in royal cabin	Bath water (23 Dec.)	Not isolated		

Lp1 and Lp5; *L. pneumophila* serogroups 1 and 5 respectively. Genomic type A, A′, A″ and B; PFGE patterns shown in lanes 1, 5, 7, and 14 in Figure (*a*) respectively.

sg 5 isolate from different origins showed a unique fingerprint, corresponding to the results of a previous report on a cluster of nosocomial Legionnaires' disease caused by *L. pneumophila* sg 5 [14], while the clinical and the environmental isolates in this outbreak showed indistinguishable or quite similar PFGE patterns.

DISCUSSION

Legionnaires' disease has been linked to passenger-ship cruises in several reports, but the source of the infection was clearly demonstrated only in two clusters [5, 6]. In the present cluster, we established that the source of infection was the ship's spa baths rather than the cabin's shower heads or the royal cabin's bath water. Cultures of spa water samples, a strainer swab, and natural stones in filters of the spas yielded *L. pneumophila* sg 1 and sg 5. By analysis with PFGE, the *L. pneumophila* sg 5 isolates from one patient were indistinguishable from the spa water isolates. All three cases were urinary antigen positive. One male patient's illness was confirmed to be due to *L. pneumophila* sg 5 infection by culture; the other male patient's illness was presumed to be due to *L. pneumophila* sg 5 infection because only *L. pneumophila* sg 5 was detected in the men's spa. Whether the causative agent of the female's pneumonia belonged to sg 1 or sg 5 could not be determined because antigen of sg 5 can cross-react with that of sg 1 in the urine antigen tests. Our results of positive urines and negative cultures (cases 2 and 3) are not rare. Generally, obtaining an adequate sputum specimen from patients with Legionnaires' disease is difficult and sensitivity is much higher in urinary antigen detection than in culture [15]. Ruf et al. reported that only two respiratory secretions were culture-positive among 13 urine-positive pneumonia patients [16]. In most situations, the use of both the urinary antigen test plus sputum culture is the best combination.

This cluster has relevant clinical implications for physicians. First, community physicians should always inquire about recent travel as part of their patient's history and consider the possibility of legionellosis, because the disease is often overlooked as a cause of community-acquired pneumonia [5]. Second, urinary antigen detection is a rapid and easy test and can detect most cases of legionellosis caused by *L. pneumophila* sg 1 and others as shown in this outbreak. However, without isolation of clinical strains, the source of infection cannot be definitely confirmed [1]. Therefore, clinical specimens for *Legionella* isolation should always be cultured.

The results of this investigation have broad public health implications. Recently, maintenance of spa baths and adherence to sanitation standards have been advocated for prevention of Legionnaires' disease in Japan and other countries. Such methodology

is now common at hotels and bathhouses after some outbreaks of Legionnaires' disease were attributed to spa water [17, 18], but not yet on ships. The cruise company had never performed surveillance for *Legionella* in the ship; the surveillance on 23 December was performed for the first time after inquiries by a regional public health centre about the probability of *Legionella* contamination. In the Japanese guidelines established by the Ministry of Health, Labour and Welfare, legionellae should not be detectable in bath water, with a sensitivity threshold of 10 c.f.u./100 ml. Exact requirements vary from prefecture to prefecture, but generally any detection of legionellae must be reported to a public health centre, which will require disinfection and retesting or closure.

Given the fact that the spas were implicated as the source of infection on this cruise ship, the implementation of preventive measures is imperative. Cruise-ship companies must be aware of the risk of legionellosis associated with the presence of legionellae in the ships' spas, and must ensure that their drains and pipework are as short as possible for easier cleaning. Moreover, they must maintain all spa apparatus fastidiously. In this cluster, *Legionella* contamination of spa water occurred in spite of the water being changed twice a day during the cruises. One reason might be the porous natural stones in filters where biofilms harbouring legionellae and other microbes are formed. Porous natural stones or ceramic balls in filters maintained poorly are so dangerous that they should be discarded. Extracts from natural stone *Maifanshi* and ceramic balls in domestic spa filters [19] and a slurry of water and sand taken from a whirlpool spa sand filter [6] harboured far more legionellae than bath water. The current most common ways of disinfecting them include superheat and hyperchlorination. Another reason is the presence of about 200 l of residual water in pipework between a heater on one floor and a bathtub several floors higher without appropriate drains. Such structural defects in spa design teach us lessons. Although the chlorine concentration of the indoor spas was set to 4 mg/l with automatic dosing devices, the actual concentration of free available chlorine residuals in the spa water had not been measured and was presumed to have been much lower than that in the guidelines of the Ministry of Health, Labour and Welfare (0·2–0·4 mg/l, $<1{\cdot}0$ mg/l).

The concentration of *Legionella* spp. in water specimens obtained from the ship's indoor spa water ranged from 80 to 15000 c.f.u./100 ml, a concentration comparable to those of previous large outbreaks in spas [18, 20] ($8{\cdot}4 \times 10^3$ c.f.u./100 ml and $8{\cdot}0 \times 10^3$ c.f.u./100 ml). Legionnaires' disease is an opportunistic infection and frequently affects people who are elderly, smoke cigarettes, or have other underlying disease [21]. Our three cases were all 70 years or older, and the index case was a heavy smoker with mild emphysema. In this cluster, however, there were few confirmed cases of Legionnaires' disease although many elderly people participated in these cruises. One probable explanation is that the indoor spas have no air compressors and therefore fewer aerosols. Another explanation may be that physicians did not order laboratory testing for the diagnosis of suspected cases.

In conclusion, this report is the first documented cluster of *L. pneumophila* sg 5 infection associated with a cruise ship and is the first cluster of Legionnaires' disease associated with a cruise ship in Japan. Since legionellosis cases associated with cruise ships have probably been overlooked, and advanced age is a risk factor for the disease [21], it is necessary to pay close attention to the hygiene of water supply systems, air-conditioning apparatus, and especially spas in any cruise ship where elderly people are likely to be passengers. We strongly recommend that porous, incompletely washable stones should not be used in a spa system.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Benin AL, Benson RF, Besser RE.** Trends in legionnaires disease, 1980–1998: declining mortality and new patterns of diagnosis. Clin Infect Dis 2002; **35**: 1039–1046.

2. **National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare.** Legionellosis, April 1999–December 2002, Japan. IASR Infectious Agents Surveillance Report 2003; 24: 27–28 (http://idsc.nih.go.jp/iasr/24/276/tpc276.html). Accessed 3 Aug. 2005.
3. **Rowbotham TJ.** Legionellosis associated with ships: 1977 to 1997. Commun Dis Public Health 1998; **1**: 146–151.
4. **Jalaludin BB, Nguyen OTK, Goldthorpe IC, Chiew RF.** Legionnaires' disease: co-infection with *Legionella pneumophila* serogroups 1 and 5. Med J Aust 1997; **166**: 277–278.
5. **Castellani Pastoris M, Lo Monaco R, Goldoni P, et al.** Legionnaires' disease on a cruise ship linked to the water supply system: clinical and public health implications. Clin Infect Dis 1999; **28**: 33–38.
6. **Jernigan DB, Hofmann J, Cetron MS, et al.** Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. Lancet 1996; **347**: 494–499.
7. **Kobayashi A, Yamamoto Y, Chou S, Hashimoto S.** Severe *Legionella pneumophila* pneumonia associated with the public bath on a cruise ship in Japan. J Anesth 2004; **18**: 129–131.
8. **Sugishita Y.** Legionnaires' disease on a cruise ship to and from Taiwan. Case 2 – Tokyo [in Japanese]. IASR Infectious Agents Surveillance Report 2004; **25**: 40–41.
9. **Takemoto M, Gonda H, Oishi T, Yamamoto K, Ikenouchi N.** A case of legionellosis on a domestic cruise ship [in Japanese]. IASR Infectious Agents Surveillance Report 2004; **25**: 41–42.
10. **Li GS, Wang JX, Jing L.** The anti-fluoride effects of Zhonghua Maifanshi. An experimental study. Fluoride 1990; **23**: 37–42.
11. **Okuda K, Ikedo M, Yabuuchi E.** Wadowsky-Yee-Okuda (WYO) medium: a new selective medium for the isolation of legionellae strains from environmental water specimens [in Japanese]. Kansenshogaku Zasshi 1984; **58**: 1073–1082.
12. **Amemura-Maekawa J, Kura F, Watanabe H, Gondaira F, Sugiyama J.** Analysis of *Legionella pneumophila* serogroup 1 isolates in Japan by using pulsed-field gel electrophoresis and monoclonal antibodies. In: Marre R, Abu Kwaik Y, Bartlett C, et al., eds. *Legionella*. Washington, DC: ASM Press, 2002: 302–304.
13. **Kuroki T, Sata S, Yamai S, Yagita K, Katsube Y, Endo T.** Occurrence of free-living amoebae and *Legionella* in whirlpool baths [in Japanese]. Kansenshogaku Zasshi 1998; **72**: 1056–1063.
14. **Chang FY, Jacobs SL, Colodny SM, Stout JE, Yu VL.** Nosocomial Legionnaires' disease caused by *Legionella pneumophila* serogroup 5: laboratory and epidemiologic implications. J Infect Dis 1996; **174**: 1116–1119.
15. **Murdoch DR.** Diagnosis of *Legionella* infection. Clin Infect Dis 2003; **36**: 64–69.
16. **Ruf B, Schurmann D, Horbach I, Fehrenbach FJ, Pohle HD.** Prevalence and diagnosis of *Legionella* pneumonia: a 3-year prospective study with emphasis on application of urinary antigen detection. J Infect Dis 1990; **162**: 1341–1348.
17. **Sugiyama K, Nishio T, Goda Y, et al.** An outbreak of legionellosis linked to bath water circulating through a filter at a spa resort, March–April 2000 – Shizuoka. IASR Infectious Agents Surveillance Report 2000; 21: 188 (http://idsc.nih.go.jp/iasr/21/247/de2471.html). Accessed 3 Aug. 2005.
18. **Nakamura H, Yagyu H, Kishi K, et al.** A large outbreak of legionnaires' disease due to an inadequate circulating and filtration system for bath water–epidemiologic manifestations. Intern Med 2003; **42**: 806–811.
19. **Takahashi T, Yabuuchi E, Endo T, Furuhata K.** Sanitary problems of 24 h-bath and administrative countermeasures for them [in Japanese]. Jap J Environ Infect 1998; **13**: 129–136.
20. **Fallon RJ, Rowbotham TJ.** Microbiological investigations into an outbreak of Pontiac fever due to *Legionella micdadei* associated with use of a whirlpool. J Clin Pathol 1990; **43**: 479–483.
21. **Marston BJ, Lipman HB, Breiman RF.** Surveillance for legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med 1994; **154**: 2417–2422.
22. **Fujita I, Tsuboi H, Ohotsuka M, et al.** *Legionella dumoffii* and *Legionella pneumophila* serogroup 5 isolated from 2 cases of fulminant pneumonia [in Japanese]. Kansenshogaku Zasshi 1989; **63**: 801–810.

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Genotyping of *Giardia intestinalis* from domestic and wild animals in Japan using glutamete dehydrogenase gene sequencing

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Abstract

To determine the genotypes of *Giardia intestinalis* from domestic and wild animals in Japan, *Giardia* isolates obtained from feces of 24 dogs kept in households and breeding kennels, three companion cats, five dairy calves and three wild monkeys, *Macaca fuscata*, were genotyped using the 177 bp sequence of the glutamete dehydrogenase gene (gdh). The genotypes were assemblages A, C, D or A/D for dog isolates, Assemblage F for cat isolates, assemblages A or E for calf isolates and assemblage B for monkey isolates. This is the first report on the genotypes of *Giardia* isolates from cats, calves and wild monkeys in Japan.

Keywords: *Giardia intestinalis*; Dog; Cat; Cattle; Wild monkey; Genotype

1. Introduction

Giardia intestinalis (syn. *G. duodenalis*, *G. lamblia*) is the most common intestinal parasite of humans as well as of domestic and wild animals. Although *G. intestinalis* isolates from different host species are morphologically indistinguishable from each other, they have been grouped into genotypes on the basis of molecular characteristics. *Giardia* isolates from humans have exclusively shown one of two major genotypes referred as to assemblages A and B (Mayrhofer et al., 1995; Monis et al., 1996), which coincide with the formerly

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used terms Polish and Belgian in Europe (Homan et al., 1992) and groups 1–3 in North America (Nash, 1995), respectively. Assemblages A and B have also been found in isolates from domestic and wild animals, such as dogs, cats, cattle, pigs, slow loris, siamang, beavers, white-tailed deer and so on, suggesting that these assemblages have zoonotic potential. On the other hand, *Giardia* isolates from animals revealed some genotypes that differed from assemblages A and B. Assemblages C and D in dogs isolates, assemblage E in cattle isolates and assemblages F and G in cat and rat isolates, respectively (Monis et al., 1996, 1999). These animal-derived genotypes seem to be each host-specific and non-zoonotic (Thompson, 2000).

In Japan, *Giardia* infection has been found in 14.6–22.9% of dogs kept in households and breeding kennels (Itoh et al., 2001, 2004) and 31.2% of companion cats (our unpublished data). Further, *Giardia* infection has also been found in 26.7% of wild monkeys, *Macaca fuscata*, in Shimokita Peninsula, and in 13.8% in wild serows, *Capricornis crispus*, in the north part of Japan (our unpublished data). There is only one report on the genotype of *Giardia* isolated from four dogs (Abe et al., 2003), though genotyping is of importance in view of public health for the above-mentioned reasons. In the present study, we clarified the genotypes of *G. intestinalis* isolated from domestic dogs and cats, dairy calves and wild monkeys in Japan using glutamete dehydrogenase (gdh) gene sequencing.

2. Materials and methods

2.1. Source of isolates

G. intestinalis isolates were obtained from feces of seven dogs (D1–D7) kept in households in Aomori Prefecture and 17 dogs (D8–D24) at six breeding kennels (a–f) in Aomori, Akita, Nagano, Niigata and Tokyo, three cats (Ct1–Ct3) in households in Aomori, five calves (Cf1–Cf5) from two farms (g and h) in Iwate and three wild monkeys, *Macaca fuscata* (M1–M3), in Shimokita Peninsula.

2.2. Extraction of genomic DNA

Giardia cysts were collected from the feces using centrifugal sedimentation in sucrose solution of specific gravity 1.21 and/or a Dynabeads anti-*Giardia* kit (Dynal A.S, Oslo, Norway) and were kept at 4 or −80 °C until use. Genomic DNA was extracted as described by Kuhn et al. (2002). Briefly, *Giardia* cysts were suspended in a mixture of 75 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and 25 μl of 10% SDS and incubated at 37 °C for 24 h. The suspension was treated with phenol/chloroform, and DNA was precipitated with chilled ethanol. The dried DNA was suspended in 10 μl MQ and used as a template for a nested PCR.

2.3. DNA amplification by nested PCR and sequencing

A DNA fragment (about 220 bp) of the gdh gene was amplified using nested PCR with primer sets of GDH1 and GDH4 (Homan et al., 1998) in the first round of PCR and GDHF3 and GDHB5 (Abe et al., 2003) in the second round of PCR. An additional primer set of gdh 1f 2nd (forward), AGGATGCTT-GAGCCGGAGCG and gdh 4r 2nd (reverse), GGA-TACTTNTCCYTGAACTC, was also used in the first PCR. Amplification was performed in a total reaction volume of 25 μl containing template DNA and the following PCR mix: 5 × Go*Taq*™ Reaction buffer, 0.2 mM dNTPs, 1.25 units of Go*Taq*™ DNA polymerase (Promega, Madison, USA) and 25 pmol of each primer. Three microlitre of the solution after the first PCR was used as a template for the second PCR. The conditions of the first and second rounds of PCR were 94 °C for 3 min initially, then 40 cycles of 94 °C for 30 s, 50 or 55 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 7 min. The reactions were performed on a GeneAmp PCR System 2700 (PE Applied Biosystems, Norwalk, USA). The products of the second PCR were visualized by electrophoresis in 1.2% agarose gel with ethidium bromide and sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Kit ver. 3.1 (PE Applied Biosystems) and the same primer set as that used in the second PCR. Sequencing reactions were analyzed on a 3100-Avant Genetic Analyzer (PE Applied Biosystems). Each DNA sample was sequenced at least two times using both forward and reverse primers. The DNAsis program (ver. 3.2, Hitachi Software, Tokyo) was used to edit sequences. Miscalled bases were corrected by analyzing chromatogram peaks and