Elimination of P. Aeruginosa in Mice by Treatment with Chlorine, and the use of Microbiological and PCR Analyses

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Summary

Unwanted micro-organisms in mice can jeopardize experimental protocols and should be eliminated if a colony becomes infected. The opportunistic bacterium *Pseudomonas aeruginosa* has been shown to cause infection in mice. In order to eliminate this bacterium from a specific-pathogen-free full barrier mouse area at our animal facility we treated mice with 7 ppm chlorine for 7 weeks and then with 10 ppm chlorine in the drinking water. The *P. aeruginosa* status of mice was examined by agar culture and PCR. The results show that 6 months after commencement of the treatment the colony was free of *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is a gram-negative, non-spore-forming, motile, aerobic bacterium belonging to the family Pseudomonadaceae. It is found in moist, warm environments and can often be isolated from soil, water, sewage, and occasionally from human skin. It was isolated in the nasopharynx and the lower digestive tract of laboratory mice and is considered to be an opportunistic agent in immune-deficient, immunosuppressed or stressed *(Urano and Maejima, 1978; Brownstein, 1978)* mice. Such mice are septicaemic, show a hunched posture, rough hair coat, emaciation and torticollis (head tilting and circling), for example, due to infection and inflammation of the inner or middle ear *(Suckow et al., 2001)*. The bacterium is resistant to mechanical cleansing and flushing, to disinfectants and to antibiotics. In a mouse facility, the normal route of infection is orally via drinking water *(http://www.criver.com/research_models_and_services/research_animal_diagnostics/LAD_literature.html, 1997)*.

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The standard method for detecting *P. aeruginosa* is by agar culture. Recently, a PCR (Polymerase Chain Reaction) method was developed to detect *P. aeruginosa* using two sequence-specific targets; the outer membrane protein (oprL) gene locus and the endotoxin A (ETA) gene locus *(Xu et al., 2004)*. OprL is an outer membrane lipoprotein which has been implicated in efflux transport systems, as well as affecting cell permeability *(De Vos et al., 1997)*. ETA is produced by the majority of *P. aeruginosa* strains and can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2, similarly to diphtheria toxin *(Khan and Cerniglia, 1994)*. The PCR with the oprL locus was reported to be more sensitive than that with the ETA locus *(Xu et al., 2004)*.

In a specific-pathogen-free (SPF) full barrier area in our mouse facility, 4 months after re-population with lactobacillus re-associated germ-free mice, *P. aeruginosa* was detected in sentinels. A previous report showed that treatment of mice with chlorine in the drinking water eliminated an experimental *P. aeruginosa* infection in mice *(Homberger et al., 1993)*. Furthermore, no toxic effects were observed at the concentrations used.

We report here the elimination of *P. aeruginosa* in mice by treatment with chlorine within a treatment

period of 6 months, as evidenced by absence of the bacterium in subsequent examinations. After cessation of chlorine treatment in a segregated colony follow-up studies using standard agar culture and PCR with fecal pellets for 12 months indicated eradication of *P. aeruginosa*.

Materials and Methods

Mice, husbandry and health monitoring of the colony

To re-populate a new full-barrier mouse area in 2003, 3 male and 5 female germ-free NMRI mice were re-associated with lactobacillus. The colony was kept in individually ventilated cages (IVCs, VentiRacks™; BioZone, Margate, UK) at a temperature of 20 to 24°C, humidity of 50 to 60%, 20 air exchanges per hour and a 12/12-hour light/dark cycle. Wood shavings (Altromin, Lage, Germany) were provided as bedding. The mice were fed a standardized mouse diet (1314, Altromin) and provided drinking water ad libitum.

Before entering a mouse room, staff showered, wore a clean gown, disposable gloves, hairnets and face masks. During routine weekly changes of cages including lids, wire bars and water bottles in class II laminar flow changing stations the mice were transferred to new cages with forceps padded with silicone tubing. Forceps were disinfected after each cage change with 70% ethanol. All materials, including IVCs, macrolon cages, lids, feeders, bottles, bedding and water were autoclaved before use.

After expansion of the NMRI colony in IVCs, retired breeding pairs were taken for microbiological screening. During expansion of the NMRI colony, embryo transfers (ET) with Swiss embryos were performed to establish an SPF Swiss colony. When the Swiss colony was large enough and the absence of all relevant micro-organisms had been confirmed, the NMRI colony was no longer bred and eventually culled. Mice of the Swiss colony were held in filter-topped Type II macrolon cages under the conditions described above and used as sentinels or ET recipients.

Sentinel-based microbiological monitoring was carried out every 6 weeks using 6- to 8-week old Swiss males as described *(Brielmeier et al., 2006)*. Briefly, aliquots of approximately 5 cm^3 of soiled bedding were taken from each used cage on a rack. These aliquots were mixed in a sterile box with an equivalent amount of new sterile bedding, and the resultant mixture was distributed to the sentinel cage of the same rack. The sentinels were kept on soiled bedding for a period of 12 weeks and then health monitored. All ET recipients were kept singly in IVCs prior to microbiological screening at 6 to 9 weeks post ET. All animal manipulations were performed in a class II laminar flow biological safety cabinet (Heraeus Instruments GmbH, Munich, Germany).

The microbiological examinations were performed according to the annual standard recommended by FELASA (Federation of Laboratory Animal Science Associations) *(Nicklas et al., 2002)* with the addition of Leptospira serogroups, ballum, canicola, hebdomadis and icterohaemorrhagiae, K virus, Lactate dehydrogenase virus, Polyoma virus, Mouse thymic virus, Hantaviruses *(Kraft et al., 1994)* and, since October 2006, murine norovirus (MNV).

The time points of health monitoring and the mice that were screened over a period of 4 years using agar culture and PCR analyses are shown in Table 1. At each time point, with few exceptions, 2 sentinels from each of 4 rooms were monitored. The number of ET recipients varied between 0 and 58. In addition, mice from 43 cages were examined at time point 22. Health monitoring of approximately 3,000 mice from time point 2 to 18 (approximately 25 months) revealed the presence of *P. aeruginosa* in 43 from 174 sentinels and ET recipients (24.7 %). With the exception of *P. aeruginosa*, the mice were found consistently negative for all of the above-mentioned infectious agents.

Treatment of drinking water with chlorine

Drinking water for the mice was prepared by filtering tap water with a 2-step (0.45 µm – 0.2 µm) sterile filter capsule (Sartobran P, Sartorius, Göttingen, Germany) which were autoclaved after each use. Just after time point 18, all mice were treated with

Table 1. Detection of *Pseudomonas aeruginosa* in mice using agar culture and PCR analyses

Time point		Agar culture			PCR		
	Week No.	Sentinel	Embryo Transfer Recipients	Total	Sentinel	Embryo Transfer Recipients	Total
1	1	0/4	0/0	0/4	n.d.*	n.d.	n.d.
2	10	3/4	n.d.	3/4	n.d.	n.d.	n.d.
3	17	2/2	3/3	5/5	n.d.	n.d.	n.d.
4	25	2/2	1/1	3/3	n.d.	n.d.	n.d.
5	31	2/6	1/6	3/12	n.d.	n.d.	n.d.
6	36	1/6	0/0	1/6	n.d.	n.d.	n.d.
7	45	3/6	3/6	6/12	n.d.	n.d.	n.d.
8	50	5/8	3/4	8/12	n.d.	n.d.	n.d.
9	57	0/8	0/5	0/13	n.d.	n.d.	n.d.
10	61	0/6	0/3	0/9	n.d.	n.d.	n.d.
11	69	0/8	1/4	1/12	n.d.	n.d.	n.d.
12	76	0/8	0/1	0/9	n.d.	n.d.	n.d.
13	80	0/8	n.d.	0/8	n.d.	n.d.	n.d.
14	87	0/8	0/6	0/14	n.d.	n.d.	n.d.
15	92	0/8	1/3	1/11	n.d.	n.d.	n.d.
16	100	6/8	2/6	8/14	n.d.	n.d.	n.d.
17	106	2/8	n.d.	2/8	n.d.	n.d.	n.d.
18	112	2/8	0/10	2/18	n.d.	n.d.	n.d.
19	119	1/10	3/15	4/25	n.d.	n.d.	n.d.
20	126	4/8	n.d.	4/8	4/8	n.d.	4/8
21	134	0/8	0/12	0/20	4/8	3/12	7/20
22	138	0/8	n.d.	0/8	0/8	n.d.	0/8
23	146	0/8	0/3	0/11	0/8	0/3	0/11
24	151	0/8	0/2	0/10	0/8	0/2	0/10
25	157	0/8	0/5	0/13	0/8	0/5	0/13
26	163	0/8	0/0	0/8	0/8	0/0	0/8
27	169	0/8	0/2	0/10	0/8	0/2	0/10
28	173	0/8	0/0	0/8	n.d.	n.d.	n.d.
29	180	0/8	0/5	0/13	0/8	0/5	0/13
30	188	0/8	0/6	0/14	n.d.	n.d.	n.d.
31	195	0/8	0/58	0/66	0/8	n.d.	0/8
32	204	0/8	0/53	0/61	0/8	n.d.	0/8
33	210	0/8	0/43	0/51	0/8	n.d.	0/8

*n.d.: not done

chlorine in the drinking water according to a previous report *(Homberger et al., 1993)* to eliminate the *P. aeruginosa* infection. The chlorinated water was prepared freshly each week in a sterile container and aliquoted in bottles. Sodium hypochlorite (13 %) was obtained from Hedinger GmbH and Co. (Stuttgart, Germany). The mice were given 7 ppm chlorine for 7 weeks just after time point 19 where *P. aeruginosa* was detected, followed by 10 ppm ad libitum at time points 20 to 33. Chlorinated water was readily accepted at the concentrations used without any noticeable effects.

Health monitoring after chlorine treatment

In addition to necropsy and microbiological analyses carried out with sentinels and ET recipients from time point 1 to time points 20 and 19, respectively, fecal pellets from all mice were also analysed by PCR as described below. To determine if mice would become positive again for *P. aeruginosa* when the chlorine treatment was discontinued after both agar culture and PCR had revealed the absence of *P. aeruginosa,* a colony of mice was segregated at time point 21 and given chlorine-free water from time points 25 to 33. The colony consisted of 5 cages of males, 5 cages of females (each cage containing 5 mice), and 15 singly-held males and 15 singly-held females. The mice were kept under filter tops under the conditions described above. Fecal pellets from each cage were analysed by PCR.

Microbiological detection of P. aeruginosa

At the time of examination, the mice were euthanised with 0.2 ml of anaesthetic intraperitoneally (33 mg/mL thiopental-sodium in 0.9% sodium chloride). Collection of samples was performed using full aseptic techniques *(Needham, 2000; Kraft et al., 1994)*. Swabs from the throat, trachea, vagina, caecum and sections from the lungs were plated out onto Columbia Blood Agar (BioMerieux, France) supplemented with 5% (v/v) defibrinated horse blood and MacConkey agar (BioMerieux) and incubated at 37°C for 24 hours. *P. aeruginosa* colonies were diagnosed by growth appearance, their green color and odour and confirmed by API 20E test strips (BioMerieux).

PCR detection of P. aeruginosa

The PCR used was a modification of the method reported by Xu et al. *(Xu et al., 2004)*. Fecal pellets were collected from cages with 2 sentinels, from cages with recipients and from cages with group-housed or singly-housed mice, and analysed. DNA from 180-190 mg fecal pellets (approximately 10 pellets) was extracted using a Qiamp DNA Stool Mini-Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. DNA was eluted from the column in 100 µl of AE buffer provided with the kit. Two different primers (Metabion, Martinsried, Germany) were used as previously described *(Xu et al., 2004)*. The first was the ExoA primer: f: 5`-GAC AAC GCC CTC AGC ATC ACC AGC-3', r: 5'-CGC TGG CCC ATT CGC TCC AGC GCT-3'and the second was the oprL primer: f: 5'-ATG GAA ATG CTG AAA TTC GGC-3', r: 5'-CTT CTT CAG CTC GAC GCG ACG-3'. The PCR with 1 µl DNA was performed in a final volume of 25 µl with 3.5 µl 10x PCR buffer (Invitrogen™ Karlsruhe), 1.6 µl (50 mM) $MgCl_2$ (Invitrogen™), 1 µl deoxynucleoside triphosphate mix (10 mM each, MBI Fermentas, St- Leon-Rot, Germany), 1 µl (mix of each single primer at 5 µM), 0.3 µl Platinum Taq DNA Polymerase (5U/µl, Invitrogen™) and 15.6 µl dH_2O. Using the ExoA primers, the PCRs started with an initial denaturation step at 96°C for 5 min followed by 40 cycles each of 96°C (60 sec), 72°C (60 sec) and 72°C (60 sec). The last cycle was followed by a 10-min extension period at 72°C. With the oprL primer, the conditions were the same as for the ExoA primers except that the annealing temperature was 55°C. Ten microliters of the PCR product were mixed with 2 µl loading buffer (MBI Fermentas), electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, visualized under UV light and photographed. The size of the amplicon was 396 bp and 504 bp for the ExoA and oprL primers, respectively.

Results

The results of chlorine treatment of the mice and *P. aeruginosa* detection at the 33 time points over a period of 4 years are presented in Table 1. The distribution of *P. aeruginosa* in the different organs from the positive mice is shown in Table 2. From a total of 500 sentinels and ET recipients that were examined by agar culture, 51 (10.2 %) were positive for *P. aeruginosa* in at least one organ between time points 2 and 20. The percentage of sentinels that were positive from 238 was 13.9 %. From 262 ET recipients, 18 were positive (6.9 %). All 51 mice were positive in the caecum while only one was positive in the vagina of an ET recipient.

Table 2. Detection of *Pseudomonas aeruginosa* in organs of 51 mice tested positive by agar culture

Organ	Sentinels	Embryo Transfer	Total
Throat	17	10	27
Trachea	1	2	3
Vagina	0	1	1
Caecum	31	20	51
Lung	3	3	6

Based on the agar culture data starting at time point 19, 1 from 10 sentinels and 3 from 15 recipients were positive for *P. aeruginosa*. At time point 20, 4 from 8 sentinel mice were positive. At time point 21, none of 8 sentinels was positive using agar culture but 4 were positive by PCR. At time points 22 and 23, all 16 sentinels were negative using both methods. With respect to the ET recipients, at time point 21, none of the 12 was positive for *P. aeruginosa* by agar culture but 3 were positive by PCR. At time point 23, none of 3 recipients was positive for *P. aeruginosa* using both methods. In addition, fecal pellets from 43 breeding pairs tested negative for *P. aeruginosa* using PCR at time point 22. At the end of the experiment at time point 33, *P. aeruginosa* was not detected in fecal pellets by either agar culture or PCR analyses from the mice that were given chlorine-free water as of time point 25. In addition, all sentinels and ET recipients examined from time points 22-23 were free of *P. aeruginosa*.

At time point 21, the Oprl and the ExoA primers detected 4/8 samples and 1/8 samples from sentinels and 0/12 samples and 3/12 samples from ET recipients as positive, respectively. PCR products were sequenced, confirming the presence of *P. aeruginosa*.

Discussion

Treatment with chlorine led to the elimination of *P. aeruginosa* in an SPF breeding area within 6 months, in contradiction to a previous report which stated that chlorine treatment did not eliminate *P. aeruginosa (Jacoby and Fox, 1984)*. After treating mice with 7 ppm for 7 weeks and still detecting the bacterium by agar culture, in contrast to a previous report *(Homberger et al., 1993)*, we increased the chlorine concentration to 10 ppm which led to elimination. Mice given 10 ppm chlorine readily drank the chlorinated water unlike previous observations *(Homberger et al., 1993)*. Furthermore, no side-effects were observed and water uptake as well as the reproductive performance of the mice were unchanged. Others reported that mice treated with 6 ppm and 8 ppm chlorine for 8 days cleared the experimental infection *(Homberger et al., 1993)*. However, in this report, mice were treated for a total of 6 months before *P. aeruginosa* was no longer detected in the entire breeding colony of approximately 3,000 mice.

During treatment of mice at our facility, we did not measure the level of chlorine in the water bottles at the end of the week. A decrease in the concentration caused by room temperature, pH or dissolved minerals *(Butterfeld et al., 1943)* may be the reason for the unexpected long duration to clearance. Another plausible reason could be the fact that mice were often kept in groups of 5 in the same cage. Positive mice could have re-infected those that had already cleared the infection via contamination of the bottles and/or coprophagy. Differences in observations

could also be attributed to the natural infection, as in the present study, in contrast to the previously reported experimental infection *(Homberger et al., 1993)*.

Human-to-animal transmission of *P. aeruginosa* has been documented *(Van der Waaij et al., 1963)*. However, the animal caretakers and the technicians in our SPF mouse area were found negative for P *aeruginosa*, thus eliminating them as a direct source of the infection.

To verify results obtained with the agar cultures, the PCR was used. During our regular health monitoring at 6-week intervals, additional in-house screening is also done for Helicobacter. To this end, the DNA extracted from the fecal pellets was aliquoted and also used for detection of *P. aeruginosa*. The PCR detected more positive mice than the agar cultures only at time point 21, most probably due to non-culturable bacteria. As such, this result was not unexpected. The PCR has the advantage in that it is rapid and can detect low copy numbers of bacterium in the early stages of infection and particularly after treatment with chlorine when bacterial growth on agar is greatly impaired. At time point 22, both methods showed the absence of *P. aeruginosa* in sentinel mice and embryo transfer recipients. Although for standard health monitoring agar culture appear sufficient to detect *P. aeruginosa* when PCR is performed both primers should be used since some positive mice were detected by only one set of primers.

P. aeruginosa is reported to be an opportunistic bacterium, affecting both wild-type and immunocompromised mice. Today, there is a boom in the number of genetically-engineered mice and their immune system may be altered by the genetic modifications. Ideally, mice used for biomedical research should be free of this bacterium, especially where immunological studies are performed. We showed in this report that even if mice become infected with *P. aeruginosa*, treatment with chlorine in the drinking water and microbiological and/or PCR analyses constitute an adequate strategy to eliminate *P. aeruginosa* from a colony.

Acknowledgements

The authors wish to thank the animal caretakers and J. Kaerlein for technical assistance.

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