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Effect of hypochlorous acid solution on the eradication and prevention of *Pseudomonas aeruginosa* infection, serum biochemical variables, and cecum microbiota in rats

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Abstract: In this study, hypochlorous acid solution, a weak acid, provided as drinking water to rats, was evaluated for its ability to eradicate and prevent *Pseudomonas aeruginosa* infection, while monitoring its simultaneous effect on serum biochemical variables and microbiota in the rat cecum. The results suggest that the solution could not eliminate the bacteria in the experimentally infected rats; however, the administration of a 10-parts-per-million (ppm) hypochlorous acid solution as drinking water was effective in inhibiting horizontal spread of *P. aeruginosa* infection among cage mates. Additionally, exposure to hypochlorous solution did not have any effect on serum biochemical variables of the rat including levels of total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, lipase, amylase, urea nitrogen, total protein, calcium (Ca), phosphorus (P), sodium (Na), chlorine (Cl), except for potassium (K) levels. The most frequently isolated bacteria in the rat cecum included species belonging to *Bacteroidales*, *Lactobacillus*, *Clostridiales*, *Erysipelotrichaceae*, *Akkermansia*, *Coriobacteriales*, and *Firmicutes*. The ratio of the terminal restriction fragment length polymorphism (T-RFLP) peaks did not differ across rats administered with 5 and 10 ppm weak acid solution as compared to the control group for any of the bacteria, except for *Erysipelotrichaceae* and *Firmicutes*, where the ratio of T-RFLP peaks was higher in the 5 ppm group for *Erysipelotrichaceae* and in the 10 ppm group for *Firmicutes* than that in the control group ($P < 0.01$). The results suggest that the weak acid hypochlorous solution could not eradicate *P. aeruginosa* completely from rats. The solution was effective in preventing infection without affecting serum biochemical variables; however, some of bacterial microbiota may have changed due to administration of the solution.

Key words: microbiota, *Pseudomonas aeruginosa*, rat, T-RFLP, weak acid hypochlorous solution

Introduction

Hypochlorous acid solution, a weak acid, is used as a disinfectant in hospitals, animal facilities, and food packaging plants. The solution is composed of sodium hypo-

chlorite and hydrochloric acid in tap water, with the pH value adjusted between 6.0 and 6.4, while residual chlorine concentration is adjusted to about 60 ppm [8]. Chlorine in solution is more effective in the hypochlorous form (HClO), and HClO is more effective as a disinfect-

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tant than ClO (NaClO) [3]. HClO has been reported to be effective against various microorganisms [5], while on the other hand, the efficacy of HClO in solution is decreased possibly due to contact with organic material in the stomach and intestine, and therefore when added to drinking water for animals, it has not been proven effective in eliminating pathogens [9]. Taharaguchi *et al.* have suggested the use of weak acid hypochlorous solution for routine cleaning in animal facilities, but they also point out that the effect could be reduced due to the presence organic material [7]. The effect of weak acid hypochlorous solution on murine Norovirus in the mouse cecum has been reported to be limited [8].

P. aeruginosa is a free-living bacterium ubiquitous in the environment, including water. It is also an opportunistic pathogen, and control of *P. aeruginosa* is often problematic, especially in animal facilities housing immunocompromised animals, despite several reports being available regarding control of *P. aeruginosa* in such facilities. Nakamura *et al.* reported that a combination of three procedures—sanitation of animal facilities, removal of *P. aeruginosa*-positive animals from the facility, and supplying tap water acidified with hydrochloric acid at pH 2.5 to 3.0—was effective in eliminating *P. aeruginosa* from the animal facility [3]. Additionally, *P. aeruginosa* has been reported to be inactivated by silver in tap water [6].

In this study, we tested whether hypochlorous weak-acid solution is effective in eradicating and preventing *P. aeruginosa* infection in rats when the solution was provided as drinking water, while monitoring rat serum biochemical variables and microbiota in the rat cecum.

Materials and Methods

Rats

Female, specific-pathogen-free (SPF) Crlj:WI rats (4-week-old) used for experimental infection with *P. aeruginosa* were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan), and were free of the following pathogens: *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Streptococcus pneumoniae*, *Mycoplasma pulmonis*, *Clostridium piliforme*, Sendai virus, Pneumonia virus, Sialodacryoadenitis virus, rat min virus, rat parvovirus, Toolan H-1 virus, Kilham rat virus, reovirus, rat theilovirus, ectoparasites, pinworm, gastrointestinal protozoa, and *Pneu-*

mocystis spp.

All rats were reared under barrier conditions, and were provided commercial laboratory rat chow and autoclaved tap water *ad libitum*. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee, Teikyo University.

Preparation of the hypochlorous weak-acid solution

The solution was prepared using a CLEAN-TE, NDX-65KM-H (OSG Corp., Osaka Japan), containing sodium hypochlorite and hydrochloric acid; the pH was adjusted to between 6.0 and 6.4, and the residual chlorine concentration was approximately 60 ppm. The solution was diluted to 5 and 10 ppm in tap water.

Pathogen

Pseudomonas aeruginosa strain JIC120501 isolated from rat at the ICLAS monitoring center was used in this study.

Attempt to eliminate *P. aeruginosa* from infected rats by substituting hypochlorous weak-acid solution for drinking water (Experiment 1)

Eighteen rats were orally infected three times with $3\text{--}6 \times 10^8$ colony-forming units (CFU) of *P. aeruginosa*, once every 24 h. At 1 week post-infection of final inoculation, rat feces were cultured on NAC agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated at 37°C for 48 h to confirm the infection. After the infection was confirmed, the rats were divided to 3 groups. Each group consisted of 6 rats, housed in 2 cages (3 rats/cage \times 2 cages). One group was administered 5-ppm and the second was administered 10-ppm hypochlorous acid solution *ad libitum* as drinking water, while the final group was provided with tap water *ad libitum*. Water was supplied via a water bottle. Residual chlorine in tap water was 0.2–0.4 ppm. The solution was changed every day.

On Days 2, 4, 7, 11, 15 and 35 after hypochlorous acid solution was given as drinking water, feces were cultured as described above, and on Day 35, serum and cecum contents were collected as well to identify any changes in serum biochemical variables and cecum microflora.

Attempt to prevent *P. aeruginosa* from infected rats by substituting hypochlorous weak-acid solution for drinking water (Experiment 2)

A total of 15 rats were used, of which 5 were experi-

mentally infected through oral route with $4\text{--}6 \times 10^8$ CFU of *P. aeruginosa* once every 24 h for a total of 3 times; these comprised the infected rats. At 1 week post infection, feces samples from these infected rats were cultured and incubated at 37°C for 48 h to confirm the infection, after which an infected rat was placed with two non-infected sentinel rats in a single cage. A total of 5 such sets (1 infected and 2 sentinel rats/cage \times 5) were prepared, of which 3 cages were provided with 10-ppm hypochlorous weak-acid solution as drinking water. The solution was administered via bottles and the solution was changed two times a week. The remaining 2 cages were provided autoclaved tap water. After 6, 13, 21, 28, 35, and 49 days of cohabitation, feces samples from these rats were cultured on NAC agar plates. On Day 49 post cohabitation, sentinels were separated from the infected rats and drinking water was changed to autoclaved tap water for all 15 rats, for an additional 34 days.

Measurement of rat serum biochemical variables

Liver-related biochemistry parameters such as levels of total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, and total bilirubin; pancreas-related parameters such as levels of lipase and amylase; and kidney-related parameters such as levels of urea nitrogen, total protein, calcium (Ca), phosphorus (P), sodium (Na), chlorine (Cl), and potassium (K) were examined on Day 35 post-administration of hypochlorous acid solution using the BioMajesty 1650 (JEOL Ltd., Tokyo, Japan) automated analyzer.

Terminal restriction fragment length polymorphism (T-RFLP)

The microbiota was analyzed using polymerase chain reaction (PCR) amplification and T-RFLP analysis. DNA was isolated from cecum contents as follows: cecum contents were suspended in a solution containing 4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 9.0), and 40 mM EDTA (pH 8.0), and then lysed in Lysing Matrix E (MP Biomedicals, CA, USA) using FastPrep-24 (MP Biomedicals) homogenizer. Thereafter, DNA was extracted using phenol-chloroform method and purified with the Gel/PCRTM DNA Isolation system (VIOGENE, New Taipei City, Taiwan). Purified DNA was amplified using a TaKaRa PCR Thermal Cycler Dice (Takara Bio, Shiga, Japan) and a set of universal primers, 5' 6-FAM-labeled 341f (5'-CCTACGGGWWGCAGCAG-3') cor-

responding to nucleotides 340–356 and 516r (5'-ATMACCGCGGCTGCTGG-3') corresponding to nucleotides 517–533 of the 16S rRNA gene of *Escherichia coli* (accession No.J01859). PCR was performed in a reaction mixture containing $1 \times$ Taq buffer, $200 \mu\text{M}$ dNTPs, 3 mM MgCl_2 , $0.2 \mu\text{M}$ each of forward and reverse primers, 10 ng purified DNA, and 1 U HotStar Taq DNA Polymerase (Qiagen, Venlo, Netherlands). PCR amplification program included preheating at 94°C for 15 min, followed by 25 cycles consisting of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 10 min. Amplified DNA was verified by 3% agarose gel electrophoresis. PCR products were purified using Gel/PCRTM DNA Isolation system (VIOGENE). T-RFLP analysis was performed as previously described [1, 2] with some modifications. Purified PCR products were digested with HpyCH4III (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h. The lengths of the terminal restriction fragments (T-RFs) were determined with the standard size marker 1200 LIZ (Applied Biosystems, Carlsbad, CA, USA) using ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and GeneScan software (Applied Biosystems). The lengths of T-RFs were treated as operational taxonomic unit (OTU) based on the 16S rDNA sequences obtained from microbiota from the rat gut. A peak OTU area was identified from the peaks detected to calculate the area under each peak. The OTU was used to estimate the phylogenetic group using the database of rat gut microbiota.

Statistical analysis.

All statistical analyses were performed using R as free software for statistical computing and graphics (<http://www.r-project.org>). Hierarchical cluster analysis was performed using Euclidean distance and group average method. Principal coordinate analysis was performed using Euclidean distance and classical multidimensional scaling. The Mann-Whitney U-test was used to analyze inter-group differences between control and experimental groups [4]. Values of $P < 0.01$ were considered statistically significant.

Results

Evaluation of efficacy of hypochlorous acid solution for elimination of P. aeruginosa (Experiment 1)

As shown in Table 1, in the 5-ppm group (cages No.3 and 4), 1 out of 6 rats tested negative for *P. aeruginosa*

Table 1. Influence of WAHS* on elimination of *P. aeruginosa* from rats

Water	Cage No.	No. of <i>P. aeruginosa</i> positive rats / negative rats					
		Days post giving WAHS (cages No.1 to 4) or tap water (cage No.5 and 6)					
		2	4	7	11	15	35
WAHS (10 ppm)	1	3/3	3/3	2/3	3/3	3/3	2/3
	2	3/3	3/3	3/3	3/3	3/3	3/3
WAHS (5 ppm)	3	3/3	3/3	3/3	3/3	2/3	3/3
	4	3/3	2/3	3/3	3/3	2/3	2/3
Tap water	5	3/3	3/3	3/3	3/3	3/3	3/3
	6	3/3	3/3	3/3	3/3	3/3	3/3

*:Weak acid hypochlorous solution.

Table 2. Biochemistry level of blood in the rats

Items	Concentration of WHAS		
	10 ppm (n=6)	5 ppm (n=6)	Cont. (n=6)
T-Chol (mg/dl)	74.3 (9.9)*	80.2 (12.2)	68.3 (11.4)
ALT (IU/l)	27.5 (2.3)	25.8 (5.3)	25.7 (5.0)
AST (iU/l)	56.5 (6.7)	56.0 (4.3)	53.5 (5.1)
ALP (IU/l)	436.2 (66.4)	440.7 (93.7)	468.7 (54.9)
Albumin (g/dl)	3.6 (0.3)	3.6 (0.2)	3.6 (0.3)
T-bilirubin (mg/dl)	<0.1	<0.1	<0.1
Lipase (IU/l)	12.2 (0.8)	12.2 (0.8)	12.5 (0.5)
Amylase (IU/l)	1,573.5 (128.2)	1,489.3 (200.2)	1,595.5 (148.9)
BUN (mg/dl)	19.8 (3.2)	20.8 (4.6)	18.3 (2.2)
T-protein (g/dl)	5.7 (0.4)	5.7 (0.4)	5.7 (0.4)
Ca (mg/dl)	10.1 (0.7)	10.3 (0.7)	10.2 (0.5)
P (mg/dl)	5.7 (0.2)	5.2 (0.7)	5.5 (0.1)
Cl (μ Eq/l)	105.0 (2.0)	106.0 (2.9)	104.7 (0.5)
Na (μ Eq/l)	150.3 (2.0)	148.0 (2.3)	149.0 (0.9)
K (μ Eq/l)	3.2 (0.4) [†]	3.3 (0.2) [†]	2.7 (0.2)

[†]: Hypochlorous acid solution (10 and 5 ppm) groups were higher than that of control group ($P<0.05$). *:mean (SD). Biochemistry levels, such as total cholesterol (T-Chol), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, total (T)-bilirubin, lipase, amylase, urea nitrogen (BUN), total (T)-protein, calcium (Ca), inorganic phosphorus (P), chlorine (Cl), were examined using Bio Majesty 1650 (JEOL Ltd., Tokyo, Japan).

in feces at 4, 15, and 35 days post administration. On the other hand, in the 10-ppm group (cages No.1 and 2), 1 out of 6 rats was negative for *P. aeruginosa* in the feces at both 7 and 35 days post administration. All 6 control rats were positive for *P. aeruginosa* throughout the experiment.

Measurement of rat serum biochemical variables

Serum biochemistry results are shown in Table 2. The serum biochemical variables of rats administered with 5- (n=6) or 10-ppm (n=6) weak acid solution were same as that of control rats, except that K level of control group ($X=2.7$, $SD=0.2 \mu$ Eq/l) was lower than that in the 5- and 10-ppm groups (5 ppm; $X=3.3$, $SD=0.2 \mu$ Eq/l, 10

ppm; $X=3.2$, $SD=0.4 \mu$ Eq/l) ($P<0.05$).

T-RFLP

In this study, *Bacteroidales*, *Lactobacillus*, *Clostridiales*, *Erysipelotrichaceae*, *Akkermansia*, *Coriobacteriales*, and *Firmicutes* were detected from rats. As shown in Table 3, species belonging to *Clostridiales* and *Lactobacillus* were the most frequently isolated bacteria in all groups, and no significant differences were observed in ratios of T-RFLP peaks for these bacteria between the control rats and those administered hypochlorous solution.

Ratio of T-RFLP peaks for *Erysipelotrichaceae* in rats who received 5-ppm solution was larger ($X=14.67$,

Table 3. Listing of the most prevalent genera in caecal samples

	% Peak Area		
	Control (n=6)	5 ppm (n=6)	10 ppm (n=6)
<i>Bacteroidales</i>	8.76 ± 5.19	6.92 ± 1.97	8.05 ± 2.40
<i>Lactobacillus</i>	26.39 ± 5.72	21.54 ± 3.52	22.21 ± 3.57
<i>Clostridiales</i>	49.92 ± 3.59	52.57 ± 3.03	52.49 ± 2.55
<i>Erysipelotrichaceae</i>	9.29 ± 2.01	14.67 ± 0.61*	11.73 ± 2.63
<i>Akkermansia</i>	1.00 ± 1.00	0.20 ± 0.24	0.30 ± 0.24
<i>Coriobacteriales</i>	1.10 ± 0.08	1.15 ± 0.06	1.15 ± 0.08
<i>Firmicutes</i>	0.18 ± 0.36*	0.67 ± 0.37	0.99 ± 0.46*

*: $P < 0.01$.**Table 4.** Influence of WAHS* on prevention of *P. aeruginosa* from the infected rats to non-infected rats

Water	Cage No.	Days post giving WAHS (cages No.1 to 3) or autoclaved tap water (cages No.4 and 5)						
		rat	Pre	6	13	36	49	
WAHS* (10 ppm)	1	Infected rat	Pos.**	Pos.	Pos.	Pos.	Pos.	
		Sentinel	Neg.***	Neg.	Neg.	Neg.	Neg.	
	2	Infected rat	Pos.	Pos.	Pos.	Pos.	Pos.	
		Sentinel	Neg.	Neg.	Neg.	Neg.	Neg.	
	3	Infected rat	Pos.	Pos.	Pos.	Pos.	Pos.	
		Sentinel	Neg.	Neg.	Neg.	Neg.	Neg.	
	Autoclaved tap water	4	Infected rat	Pos.	Pos.	Pos.	Pos.	Pos.
			Sentinel	Neg.	Pos.	Pos.	Pos.	Pos.
		5	Infected rat	Pos.	Pos.	Pos.	Pos.	Pos.
Sentinel			Neg.	Pos.	Pos.	Pos.	Pos.	
		Sentinel	Neg.	Pos.	Pos.	Pos.	Pos.	

*: Weak acid hypochlorous solution. **: Positive for *P. aeruginosa*. ***: Negative for *P. aeruginosa*.

SD=0.61) than that of control group ($X=9.29$, $SD=2.01$) ($P < 0.01$). For *Firmicutes* spp., the ratio of T-RFLP peaks for rats who received 10 ppm solution ($X=0.99$, $SD=0.46$) was higher than that of control group ($X=0.18$, $SD=0.36$) ($P < 0.01$). Ratio of T-RFLP peaks for all other bacteria was similar in all groups.

Evaluation of efficacy of hypochlorous acid solution for prevention of *P. aeruginosa* (Experiment 2)

As shown in Table 4, *P. aeruginosa* was not detected in feces from the 6 sentinel rats exposed to the infected rats in the group that received the hypochlorous solution until 49 days of cohabitation, despite that *P. aeruginosa* was detected in feces of the infected rats. However, *P. aeruginosa* was detected in feces of all rats provided with autoclaved water. Bacterial counts in cecum of infected rats who received 10-ppm hypochlorous solution and autoclaved water were 3.3×10 CFU/g (feces) and

1.0×10^3 CFU/g (feces), respectively (data not shown). On Day 49 post cohabitation, sentinels were separated from the infected rats and drinking water was changed to autoclaved water for an additional 34 days, after which, *P. aeruginosa* was not detected in feces of the sentinel rats (data not shown).

Discussion

P. aeruginosa causes an opportunistic infection and the organism needs to be controlled, especially in facilities with immunocompromised animals. Environmental cleanup in such facilities is carried out using a chlorhexidine or phenolic disinfectant, and/or by supplying antibiotics, or tap water acidified with hydrochloric acid at pH 2.5 to 3.0. Recently, effectiveness of the weak acid hypochlorous solution *in vitro* on various microorganisms including *P. aeruginosa* was reported

[5, 7]. On the other hand, the solution used as disinfectant for drinking water was ineffective as the effectiveness of HClO in the weak acid hypochlorous solution is possibly decreased by contact with organic materials in the stomach and intestine, and the effect of the weak acid hypochlorous solution on murine Norovirus was limited to the small intestine [8].

In this study, we evaluated the feasibility of consuming hypochlorous solution as disinfectant for potable water to eliminate *P. aeruginosa* in experimentally infected rats and studying its influence on serum biochemistry and cecum microbiota in the rats.

As shown in the results of Experiment 1, the hypochlorous weak-acid solution did not eliminate *P. aeruginosa* from experimentally infected rats completely; however, *P. aeruginosa* was not isolated from rats on Days 7 and 35 post administration of 10 ppm solution, and on Days 4, 15 and 35 post administration of 5 ppm solution.

Results of Experiment 2 showed that horizontal infection could be prevented by providing the solution. Finally, we concluded that the hypochlorous solution used as drinking water did not eliminate *P. aeruginosa*, but was effective in preventing infection. We speculate the number of *P. aeruginosa* caused the different efficacy between elimination and prevention. In experiment 1, rats were experimentally infected with a concentrated *P. aeruginosa* suspension (3 to 6×10^8 CFU). During a spontaneous infection by *P. aeruginosa*, rats may be infected by a lower number of bacteria; therefore, the hypochlorous acid solution may be effective to prevent transfer of *P. aeruginosa* infection to cage mates.

In Experiment 2, autoclaved tap water was provided as drinking water in bottles to the control group (cages No.4 and 5) to rule out the possibility of infection to sentinel rats by *P. aeruginosa* contaminated in tap water. However, even with non-autoclaved tap water containing 0.2–0.4 ppm residual chlorine, used as drinking water, *P. aeruginosa* infection was transferred to sentinel rats within 3 days of cohabitation with infected rats (data not shown).

For practical use of the solution, the influence on rat serum biochemical variables was evaluated. Serum biochemistry parameters including function of the liver, pancreas, and kidney were compared between the control and group receiving the hypochlorous solution. There was no difference observed in serum biochemical variables in the control and 2 groups receiving different

concentrations of hypochlorous solution except for K levels, which reflect an impact on kidney function; however, other biochemical parameters such as BUN, Ca, and Na, also associated with kidney function, were normal, suggesting that the solution did not affect kidney function.

Ratio of the frequently isolated bacteria in rat cecum microbiota were compared in the control and 2 groups receiving different concentrations of hypochlorous solution. In total, 7 types of bacteria were detected. Especially for *Firmicutes* spp., the ratio remarkably increased with the concentration of the solution, suggesting that ratio of *Firmicutes* spp. in bacterial microbiota in rat was increased by the administration of hypochlorous weak-acid solution. However, the ratio of major bacteria such as *Clostridiales* and *Lactobacillus* species were not different in the control group and hypochlorous solution groups, and any clinical signs such as diarrhea and bowel disease were not observed, suggesting use of hypochlorous solution as drinking water may not affect result of animal experimentations.

In the prevention study, *P. aeruginosa* was not detected in feces from sentinel rats that received hypochlorous weak-acid solution even if these rats were exposed to *P. aeruginosa*-infected rats during the experiment. On the other hand, *P. aeruginosa* was detected in feces from sentinel rats provided with autoclaved water as soon as 6 days post exposure, suggesting that the hypochlorous weak-acid solution was useful in preventing the spread of infection in animal facilities. In this experiment, rat feces were cultured to evaluate whether the rats were infected. To confirm the results, continuous monitoring (6, 13, 36, and 49 days post hypochlorous solution administration) was performed for *P. aeruginosa* in rat feces.

In conclusion, the hypochlorous weak-acid solution could not eradicate *P. aeruginosa* completely from rats but the solution was effective to prevent infection without affecting serum biochemical variables; however, some of bacterial microbiota may have changed due to administration of the solution.

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