APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 2168–2169
0099-2240/94/$04.00+0
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Vol. 60, No. 6

Efficacy of Chemical Disinfectants against Turbot Aquareovirus

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Received 2 November 1993/Accepted 5 April 1994

The susceptibility of turbot aquareovirus to five chemical agents was examined. Treatment with 5 mg of malachite green per liter or 500 mg of iodine per liter resulted in a 90% reduction in virus titer within 1 h. Complete inactivation within 5 min was obtained with 2% formalin, 42.5% isopropanol, or 15 mg of free available chlorine per liter. Lower concentrations of chlorine were ineffective.

Strains of the genus *Aquareovirus*, which belongs to the family *Reoviridae*, have been detected in a large number of fish and shellfish (7). The increasing number of members of this group isolated from poikilothermic animals emphasizes the necessity to understand their epidemiology and ways of transmission. Nonpersistent disease control agents are needed in several phases of intensive fish culture (3, 15). The control of viral infections requires the use of chemical disinfectants to destroy the infective agent in hatcheries, tanks, ponds, effluent water, or equipment. In the present study, the susceptibility of turbot aquarcovirus (TRV) to five commercially available disinfectants was examined.

TRV, isolated from turbot (10), was used in all of the assays. Virus production was carried out on monolayers of the Chinook salmon embryo cell line CHSE-214 (6). The cells were cultured in Eagle's minimal essential medium (Gibco) supplemented with 5% fetal bovine serum (Gibco), 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Cell monolayers were inoculated with the virus at a multiplicity of infection of 0.1. When cytopathic effect was extensive, the cells were scraped off and the suspension was frozen and thawed three times. The suspensions were clarified by low-speed centrifugation (2,000 × *g* for 10 min at 4°C) and stored at −80°C until use.

The following commercially available disinfectants were used to test their ability to inactivate TRV: malachite green (2, 5, and 10 mg/liter); 4 and 0.05% solutions of commercial formalin in phosphate-buffered saline (PBS); solutions containing 0.4, 10, 15, and 125 mg of free available chlorine per liter (pH 10.6, 9.0, 8.1, and 7.7, respectively), prepared from a commercially available disinfectant containing 11% chlorine; iodine diluted in distilled water to provide solutions containing 25, 50, 100, and 1,000 mg of available iodine per liter; and 85% isopropanol. The concentrations of available chlorine were determined by a colorimetric version of the DPD (*N,N*-diethyl-*p*-phenylenediamine chlorine) method (DPD colorimetric method) (9), which allows detection of a minimum concentration of 10 μg of Cl (as Cl_2) per liter.

The assays were conducted after dilution of the initial viral inoculum (10^4 to 10^5 50% tissue culture infective doses [$TCID_{50}$]/ml) in the disinfectant (1:1). The mixture was incubated at 15°C for 1 or 6 h for malachite green and 5, 15, 30, or 60 min for the remaining disinfectant agents. After the incubation period, the chemical agents were neutralized by the addition of an equal volume of specific neutralizing solutions. The neutralizing mixture for 4 and 0.05% formalin consisted of 4% and 0.04% $Na_2S_2O_5$ in PBS, respectively. For neutralization of chlorine and iodine, 2 mM (5) and 8 mM concentrations of sodium thiosulfate, respectively, were used. The isopropanol was neutralized with distilled water. In parallel, inactivation of TRV by exposure to different pH values (3, 5, 7, 9, and 11) was evaluated. Briefly, Eagle's minimal essential medium adjusted to pH 3, 5, 7, 9, and 11 was mixed (1:1) with the viral suspension, and the mixture was incubated for 4 h at 15°C. At different times, samples were collected, and the virus titers were determined. All viral infectivity assays were carried out as triplicate microtitrations in 96-well culture plates as previously described (14). Results were recorded after 14 days of incubation at 15°C, and 50% infectivity endpoints were calculated by the method of Reed and Müench (12). Disinfectant agents, disinfectant-neutralizer mixtures, and untreated viral inoculum were used as controls.

The experimental temperature of 15°C was chosen because it is within the range of temperatures prevailing in the turbot farms in Galicia, northwestern Spain, the area where TRV has been detected.

Malachite green (1 to 2 mg/liter) was used as a bath or flush solution for 1 h (11). Our results showed that a low reduction (90%, corresponding to 1 log unit) in TRV titers occurred after treatment of viral suspensions with 5 mg of malachite green per liter for up to 24 h at 15°C (Table 1). No reduction in the titer was observed when lower concentrations of malachite green were tested for the same length of time (data not shown). This finding is in agreement with those reported for the virucidal activity of this agent against other fish viruses, confirming that such treatment does not eliminate the viral agents from the aquatic environment (1, 2, 5).

The concentration of formalin recommended for use as a disinfectant in farms is as low as 2%. We observed that

TABLE 1. Stability of TRV following treatment with malachite green and formalin at 15°C

Treatment	TRV titer (log $TCID_{50}$/ml) after exposure time of:				
	0 min	5 min	30 min	1 h	24 h
Malachite green (5 mg/liter)	4.5	NT[a]	NT	3.5	3.5
Formalin (0.025%)	4.25	4.25	4.25	4.25	NT
Formalin (2%)	4.5	0.0	0.0	0.0	NT

[a] NT, not tested.

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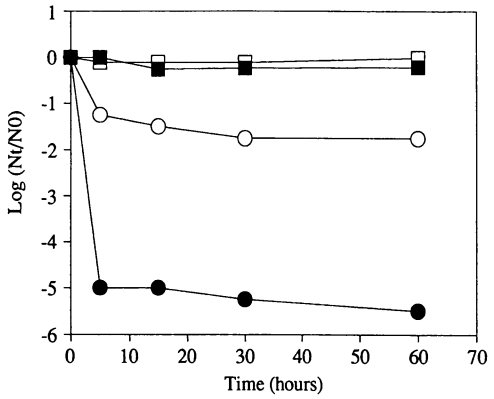

FIG. 1. Inactivation of TRV by hypochlorite at 15°C. The concentrations of available chlorine in milligrams per liter were as follows: 0.4 (□), 10 (■), 15 (○), and 125 (●). N_t is the titer of the virus at time t, and N_0 is the initial virus titer.

treatment of infective cell culture fluids with 2% formalin resulted in reduction of viral titers below the detection limits within 5 min of exposure (Table 1). A concentration of 0.025% formalin, however, resulted in no loss of infectivity. Similar results have been reported by other authors (1, 5, 8). Frerichs (5) observed a marginal loss of viral infectivity after a 6-h reaction with low concentrations of this agent. These features indicate that such low concentrations of formalin are not of practical value in eliminating infectious virus.

The effect of chlorine on the survival of TRV in cell culture fluids is shown in Fig. 1. The virus appeared to be completely inactivated following exposure to 15 mg of free available chlorine per liter for 5 min. No more than a marginal loss (no more than 99%, or 2 log units) in infectivity was observed when concentrations of 10 mg or less per liter were tested. The residual chlorine found in the suspensions at the conclusion of the incubation period (1 h) was measured. The following concentrations of available free chlorine were found: 0.05, 0.17, 2.15, and 2.9 mg/liter, from the lowest to the highest initial chlorine concentration. It could be thought that the inactivation of TRV by chlorine at the highest concentration was due to the low stability of the virus at the high pH values of the solutions used. However, as shown in Table 2, TRV was very stable at all pH values tested during the experiment. Previous studies of the effectiveness of chlorine treatment showed variable degrees of susceptibility to this agent depending on the virus assayed (1, 8, 15). Thus, Wedemeyer et al. (15) observed that as little as 0.1 mg of chlorine per liter completely inactivated infectious hematopoietic necrosis virus within 30 s. However, for total inactivation of infectious pancreatic necrosis virus, a 30-min incubation with 40 mg of chlorine per liter was necessary (8).

No significant inactivation of virus was observed with any of the concentrations of iodine tested. These findings coincide with those reported by Frerichs (5), who indicated that no loss of infectivity of two snakehead rhabdovirus strains occurred following treatment with 25 mg of iodine per liter for 30 min. In contrast, other authors (2, 8) found that 5 min of treatment with 16 mg of iodine per liter completely inactivated infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus and that 5 min of treatment with 35 mg of iodine per liter completely inactivated infectious pancreatic necrosis virus.

The effectivity of isopropanol as a disinfectant was also studied. In this experiment, TRV was completely inactivated following treatment with 42.5% isopropanol for 15 min at 15°C.

Our results clearly indicate that although chlorine and iodine are recognized as potent virucidal agents, with a wide range of applications in fishery management (4), they are not effective at inactivating TRV. In addition, chlorine does not appear to be a suitable disinfectant for the treatment of surface waters because of the production of trihalomethanes (13). Therefore, we recommend agents such as isopropanol as well as formalin as disinfectants to be used in fish farms.

TABLE 2. Stability of TRV after incubation at different pH values

Incubation time (h)	TRV titer (log $TCID_{50}$/ml) at pH:				
	3	5	7	9	11
0	5.75	5.75	5.75	5.75	5.75
1	5.75	5.75	5.5	5.5	5.25
2	5.5	5.5	5.5	5.75	5.25
3	5.25	5.5	5.25	5.5	5.0
4	5.0	5.5	5.25	5.25	4.75

This work was supported by grants MAR 89-0270 from the Comisión Interministerial de Ciencia y Techología (CICYT) and XUGA 8030389 from the Xunta de Galicia (XUGA), Santiago de Compostela, Spain. C. Rivas, I. Bandín, and C. Cepeda acknowledge the Ministerio de Educación y Ciencia of Spain for research fellowships.

We thank the anonymous reviewers for useful comments which helped to improve this article.

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