2015| Volume 1 | Issue 1 | p. 9 - 15

Ewemen Journal of Microbial Research

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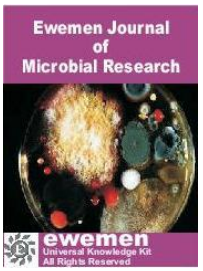

Full Length Research

BACTERICIDAL EFFECTIVENESS OF SOME COMMERCIAL DISINFECTANTS AGAINST *AEROMONAS HYDROPHILA* AND *VIBRIO VULNIFICUS*

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Received 8 August, 2015
Revised on the 10 August, 2015
Accepted 18 August, 2015

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ABSTRACT

In vitro study was carried out following the procedures, test methods of BS EN 1276. Six commercially available disinfectants (Aldekol-GDA, TH4, Biosentry Iodine, Peraclen, Virkon-S and Biosentry 904) were evaluated for their bactericidal efficacy against two bacterial pathogens (*Aeromonas hydrophila (gyrB LC012344)* and *Vibrio vulnificus*) at 20 °C. The CEN method identifies 5 min as the disinfectant contact time, additional 1, 10 and 30 min contact time was included for comparative purposes between the used disinfectants. The results revealed that:1) 1 min contact time, only 3 disinfectants passed at their recommended use dilutions (under dirty conditions) against *Aeromonas hydrophila (gyrB LC012344)* achieving microbicidal effect (ME) (log reduction) ≥ 5 (Verkon-S, Aldekol Aldekol des- Gda®, TH4); 2) after a 5 min contact time another 2 disinfectants passed and achieved 5 log reduction (ME) (Biosentry 904, Peraclen); 3) This study demonstrates the need for final verification of disinfectant efficacy by undertaking field or *in vivo* trials in the aquatic environment in which the disinfectant is intended for use to achieve reproducibility and reality of results.

Keywords*:* Aquaculture, Disinfection, Quantitative Suspension Test, *Aeromonas hydrophila, Vibrio vulnificus*.

INTRODUCTION

In aquaculture, more than another animal and food production sectors, prevention is a key issue of health maintenance and management. Like land-based farm animals, farmed fishes are subjected to diseases associated with bacterial infections. Fish culture is currently suffering from serious losses due to infectious diseases (Furones and Rodgers, 2009}. The impact of these diseases on animal health and the economics of the farm can be limited by good husbandry and by the use of appropriate prophylactic measures and antimicrobial agents. Although the capability to manage aquaculture health issues has increased tremendously in the last 30 years, the rapid and on-going development of all aquaculture sectors continuously to "raise the bar" with new challenges (Alday *et al.*, 2006).

Reduction of fish diseases is undoubtedly very important for the future success of the aquaculture industry (Johari *et al.*, 2014). Mesophilic motile *Aeromonas* spp. are normal inhabitants of soil and freshwater. Over the last 3 decades, these organisms have also emerged as opportunistic pathogens responsible for gastroenteritis, septicemia, skin and soft tissue infections and a variety of clinical syndromes in fish and humans (Jones and Wilcox, 1995). The genus *Vibrio* includes many pathogenic species to humans and fish e.g., *V. cholera, V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. anguillarum,* and *V. salmonicida* (Reed and Francis-Floyd, 2002). *Vibrio* come on the top list of pathogens with direct jeopardy to marine culture development due to high mortalities associated with their invasion to fishes (Austin and Austin, 2012).

Disinfectants are very useful, but vary in their effectiveness against specific disease organisms. Standard doses will kill many pathogens, but some may require more specific doses or contact times. (Roy *et al.,* 2012). Disinfectants are tested to ensure that they are capable of delivering the degree of protection required by the user or promised by their manufacturers or suppliers. Several factors should be included in an evaluation of a disinfectant. A prerequisite for a disinfectant is its spectrum of effectiveness against pathogens. It is imperative that disinfectants be selected after establishing their effectiveness against specific pathogens rather than adopting a broad, non-specific, unfocused, and often, the meaningless disinfectant selection criterion of "something that kills everything". Although many identical disinfectants are used in many different countries, a general internationally accepted scheme does not exist (Reybrouck, 1991). Testing of disinfectants for their microbicidal activity is, in principle, easy, but in reality quite complex. There are a myriad of factors to deal with to ensure the repeatability and reliability of the test (Staniforth, 2013; Phelps, 1911; Chick, 1908; Watson, 1908). Many countries have their own government testing laboratories with their own national standards for testing disinfectants. A disinfectant that is passed for use in one country may not necessarily pass in another, most of the microbicidal tests used for routine and research purposes are quantitative suspension tests, in which the number of survivors is determined by direct culture (Staniforth, 2013).

Within Europe, there has been a drive to standardize terminology and thus testing. CEN TC 216 is concerned with "standardization of the terminology, requirements, test methods, including potential efficacy under in-use conditions, recommendations for use and labeling in the whole field of chemical disinfection and antiseptics (BSI, 2009). The goal of this study was to examine the efficacy of common aquaculture compounds for disinfecting against two bacterial species affecting aquaculture: *Aeromonas hydrophila (*and *Vibrio vulnificus,* under laboratory conditions to provide a recommendation of the most effective compound(s) for the prevention of infection in an aquaculture setting.

MATERIALS AND METHODS

Bacterial strains

Aeromonas hydrophila (*Gyr-B LC012344*) - isolated from cultured Nile tilapia, *Oreochromis niloticus* during a disease outbreak in Fayoum governorate, Egypt, in 2012. Molecular identification was performed using the primers designed by Hu *et al.,* (2012) for detection of 1100 bp fragment of Gyr-B gene.

Vibrio vulnificus - isolated from diseased shrimp *Penaeus indicus* during a disease outbreak affecting different private fish farms in the Eldebah triangular area, Port Said, Egypt 2014. Molecular identification was carried out for detection of 437 bp fragment of cytotoxin gene.

Tested disinfectants

Commercially available six (6) disinfectants products (Table 1) chosen to represent a different range of active compounds were used. Their recommended use dilution was achieved using sterile hard water (BSI, 2009). The effectiveness of test disinfectants was assessed following the procedures of BS EN 1276 phase II step 1, BSI (2009). That is, the quantitative suspension test for the evaluation of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas — test method and requirements according to Comitè Europèèn De Normalisation (CEN) (BSI, 2009).

1. **Table 1: Selected disinfectants and their manufacturer's recommended use dilution.**

No	Disinfectant	Class of Disinfectant	Active ingredients	Used dilution	Manufacture company
1	Verkon- S®	Peroxygen compounds	Potassium peroxymonosulfate (20.4%)/ NaCl (1.5%)	1:120	DuPont™ (USA)
2	Biosentry® Iodine™	Iodine disinfectant	Hydroxypoly Iodine complex Active Iodine (1.75%)	0.16%	DuPont™ (USA)
3	Aldekol Des-Gda®	Aldehyde / QUACS combined	Glutraldehyde / QUACS	0.4%	EWABO Chemkalien, GmbH
4	TH4	TH4	Didecyl dimethyl ammonium chloride 18.75 g. Dioctyl dimethyl ammonium chloride 18.75 g. Octyl decyldimethyl ammonium chloride 37.50 g. Alkyl dimethyl benzyl ammonium chloride 50 g. Glutraldehyde 62 g.	0.5%	Sogeval, a French veterinary drug company
5	Biosentry® 904™	Quaternary ammonium compound	QUACS 24% Didecyl dimethyl ammonium chloride dimethyl benzyl ammonium chloride	0.4%	DuPont™ (USA)
6	Peraclen®	Hydrogen peroxide, per-acetic acid and acetic acid	H_2O_2 (20%) Per-acetic acid (5%) Acetic acid (10%)	1%	Henkel (Germany)

Culture media

The culture and media used for maintenance of test microorganism and for viable counts were tryptone soya agar TSA (Oxoide) for *Aeromonas hydrophila (gyrB LC012344)* and supplemented with 3% NaCl for *Vibrio vulnificus.*

Diluents

Diluents used throughout contained 0.1% tryptone (Oxide) and 0.85% sodium chloride dissolved in distilled water as described in the 1987 European Suspension Test (EST) (Anon, 1988).

Neutralization media

Neutralization solutions were prepared by using a blend mixture of Lecithin, Tween-80, Sodium thiosulphate and L-histidine. Phosphate buffer saline 0.25 N was added then steam sterilized in an autoclave for 15 mins. at 121˚C. For all disinfectants, preliminary tests were carried out to confirm the efficacy of NM as described in EN 1276, validation test 2 (BSI, 2009).

Hard water

Disinfectants were diluted using water of standard hardness prepared according to EN 1276 produced a final hardness of (375-400 ppm $CaCO_3$) in each test tube. (BSI, 2009)

Organic load

Bovine albumin and Yeast extract mixture were prepared by mixing an Equal volume of 10% solution of Bovine albumin and Yeast extract containing a final concentration of 5% albumin and 5% yeast extract.

Principle of test

The standard bactericidal activity is verified in three phases of testing:
Phase I studied whether the product, diluted in distilled water, has a basic level of activity in the absence of any organic soil. At this phase only one strain of *Staphylococcus aureus* and *Pseudomonas aeriogenosa* were used to determine basic bactericidal activity. Phase II determined the activity in stimulating use conditions with organic load and several test microorganisms, either as a suspension

test (step 1) or a surface test (step2). Phase III consisted of 'in-use' trials.

Contact time

The CEN method identifies 5 mins as the disinfectant contact time. However, additional 1, 10 and 30 mins contact time was included for comparative purposes between the used disinfectants.

Procedures

For each strain culture the test was repeated on the second day and, if the two results were at variance, a third definitive test was carried out with the overall performance of the products assessed at their in-use dilution.

Test organisms and test suspension:

i. After isolation and identification of the test organisms, strains were maintained over the long term as frozen stocks at -80˚C. Cultures used for the disinfection assays were prepared from subculture on TSA allowed to grow for 24 hrs at 25 - 28˚C and stored in a refrigerator at 5˚C until required.
ii. Subcultures were performed on at least 2 but not more than 3 occasions, from these subcultures test suspensions were prepared and diluted using tryptone-NaCl saline and number of colony forming units (cfu) were adjusted where possible to (1-5×10^8 mL^{-1}) by surface spread viable counting method.
iii. The suspensions were maintained at 20˚C ± 1˚C and used within 2 hrs.

Disinfectant test methods (phase 2 / step 1) (EN 1276)

a) Before starting the test, all reagents were equilibrated to 20˚C in a water bath, 8 mL of disinfectant test solution at manufacturers recommended use dilution previously prepared with sterile hard water was added to 1 mL of albumin/yeast mixture and mixed by vortex and left for about 30 mins before 1 mL of the test culture bacterial suspension was added.
b) One millilitre (1 mL) was removed after 1, 5, 10 and 30 mins contact time after the addition of the test culture suspension and added to 8 mL neutralization medium to which 1 mL sterile distilled water had been added. The mixture was thoroughly mixed by vortex and left at 20˚C. After a neutralization time of 5 mins for each contact time, further decimal dilutions were made in diluents as appropriate and 100 µL was inoculated and spread onto duplicate plates of appropriate media.
c) Three control procedures were carried out to ensure the validity, reproducibility and repeatability of the test in parallel for each disinfection [Control A: 8·0 mL water of standard hardness in place of the disinfectant to 1 mL of the strain suspension and 1 mL of organic substances to verify the absence of any lethal effect in the test conditions. Control B: Neutralizer (8·0 mL) and 1 mL water were added to the strain suspension and then plated out to ensure that the neutralizer had no disinfectant activity. Control C: Bacterial suspension (1mL) was added to 8.0 mL neutralized disinfectant to ensure that the disinfectant had been neutralized].
d) Viability reduction is calculated for each microorganism and test concentration using the following formula:

$$R = \frac{N * 10^{-1}}{N_a}$$

Where: $\boldsymbol{R}$ = Reduction of viability; $\boldsymbol{N}$ = bacterial counting for the initial test suspension; and $\boldsymbol{N_a}$ = bacterial counting for the test mixture at the end of the contact time. [That is, the microbicide effect (ME) was calculated so, by subtracting the log number of cfu mL^{-1} after action of the disinfectant from the log number of 0.1 cfu mL^{-1} of the test suspension].

Statistical Analysis

A paired t-test was implemented, and differences between means were considered to be significant at P-values less than 0.05.

RESULTS AND DISCUSSION

To pass the test, products must achieve a five log reduction in viable counts. Results in the listed Tables (2 & 3, and 4 & 5) showed the mean viable colony count and the mean Microbicide Effect (ME) (log reduction) of the tested disinfectants against *Aeromonas hydrophila* (*gyrB LC012344*) and *Vibrio vulnificus* respectively.

Tables (2) and (3) indicated that at 20 °C and 1 min contact time, only 3 disinfectants passed at their recommended use dilutions (under dirty conditions) against *Aeromonas hydrophila* (*gyrB LC012344*) achieving ME (log reduction) ≥ 5, (Verkon-S, Aldekol D des- Gda®, TH4), but after a 5 mins contact time

another 2 disinfectants passed and achieved ME (log reduction) ≥ 5 (Biosentry 904, Peraclen). However, Biosentry Iodine failed the test even after 30 mins contact time.

Concerning *Vibrio vulnificus* depopulation, only 3 disinfectants passed after 1 min contact time under the same conditions (Biosentry Iodine, Aldekol des-Gda and Biosentry® 904™), but all the tested disinfectants passed after a 5 mins contact time (Table 4 & 5). Each pathogen needs to be taken into consideration for effective disinfection. *Vibrio spp.* act differently from other bacterial species which may have different levels of resistance to disinfection. For example, *Mycobacterium marinum* was resistant to many disinfectants and only susceptible to Lysol® and 50% ethanol (Mainous and Smith, 2005).

Table 2: The mean viable colony count (cfu/mL) of *Aeromonas hydrophila* (*gyrB LC012344*) after contact with tested disinfectants.

Disinfectant/ contact time	Initial count	1 min.	5 min.	10 min.	30 min.
Verkon- S®	3.7×10^8	0	0	0	0
Biosentry® Iodine™	3.5×10^8	2.5×10^5	2.7×10^5	7×10^5	4.6×10^6
Aldekol Des- Gda®	2.4×10^8	0	0	0	0
TH4	2×10^8	0	0	0	0
Biosentry® 904™	2.1×10^8	5×10^3	0	0	0
Peraclen®	3.5×10^8	1×10^4	0	0	0

Biosentry® 904™, Peraclen®

Table 3: The mean Microbicide Effect (ME) (log reduction) of tested disinfectants against *Aeromonas hydrophila (gyrB LC012344).*

Disinfectant/ contact time	Initial count	ME (log reduction) after exposure			
		1 min.	5 min.	10 min.	30 min.
Verkon- S®	3.7×10^8	8.57*	8.57*	8.57*	8.57*
Biosentry® Iodine™	3.5×10^8	2.15**	2.11**	1.70**	0.88*
Aldekol Des- Gda®	2.4×10^8	8.38*	8.38*	8.38*	8.38*
TH4	2×10^8	8.3*	8.30*	8.30*	8.30*
Biosentry® 904™	2.1×10^8	4.12**	8.32*	8.32*	8.32*
Peraclen®	3.7×10^8	4.14**	8.57*	8.57*	8.57*

*Disinfectants passed of ME (log reduction) ≥ 5
** Disinfectants failed of (log reduction) <5

Table 4: The mean viable colony count (cfu/mL) of *Vibrio vulnificus* after contact with tested disinfectants.

Disinfectant/ contact time	Initial count	1 min.	5 min.	10 min.	30 min.
Verkon- S®	2.4×10^8	3×10^4	0	0	0
Biosentry® Iodine™	3.6×10^8	0	0	0	0
Aldekol Des- Gda®	3.3×10^8	0	0	0	0
TH4	3.2×10^8	3.1×10^3	0	0	0
Biosentry® 904™	3.3×10^8	0	0	0	0
Peraclen®	2.4×10^8	3.6×10^5	0	0	0

On one hand, 1 min of contact time of Verkon- S , Aldekol des- Gda and TH4 (using recommended dilution) significantly reduced the population of *Aeromonas hydrophila* (*gyrB LC012344*) with a complete reduction of the population. Biosentry Iodine however failed the test even after 30 mins contact time (Tables 2 & 3). On the other hand, 1 min contact time of Biosentry Iodine, Aldekol des- Gda and Bioentry 904 significantly reduced the population of *Vibrio vulnificus* with a complete reduction of the population (Tables 3 & 4).

The findings that Biosentry Iodine failed the test on *Aeromonas hydrophila* even after 30 mins contact time (Tables 2 & 3) is contrary to the findings of Cipriano et al. (2001) who reported *Aeromonas salmonicida* susceptibility to iodophor (povidone iodine) disinfection to reduce the incidence of disease from contaminated salmon eggs (Cipriano *et al*. 2001).

Table 5: The mean Microbicide Effect (ME) (log reduction) of tested disinfectants against *Vibrio vulnificus.*

Disinfectant/ contact time	Initial count	ME (log reduction) *Vibrio vulnificus* after exposure			
		1 min.	5 min.	10 min.	30 min.
Verkon- S®	2.4×10^8	2.90**	7.38*	7.38*	7.38*
Biosentry® Iodine™	3.6×10^8	7.56*	7.56*	7.56*	7.56*
Aldekol Des- Gda®	3.3×10^8	7.52*	7.52*	7.52*	7.52*
TH4	3.2×10^8	4.01**	7.51*	7.51*	7.51*
Biosentry® 904™	3.3×10^8	7.51*	7.51*	7.51*	7.51*
Peraclen®	2.4×10^8	1.83**	7.38*	7.38*	7.38*

*Disinfectants passed of ME (log reduction) ≥ 5
** Disinfectants failed of (log reduction) <5

Ethyl alcohol (30, 50, or 70%), benzyl-4-chlorophenol/ phenylphenol (1%), sodium hypochlorite (50, 100, 200, or 50,000 mg/L), n-alkyl dimethyl benzyl ammonium chloride (1:256), povidone iodine (50 or 100 mg/L), glutaraldehyde (2%), and potassium peroxymonosulfate/sodium chloride (1%) had been reported to be effective disinfectants, as each reduced or eliminated the number of detectable organisms within 1 min of contact time. However, neither Chloramine-T (15 mg/L) nor formalin (250 mg/L) was found to substantially reduce bacterial counts even after 60 min of contact time (Mainous and Smith, 2010).

Potassium peroxymonosulfate/ sodium chloride (Virkon-S; reformulated as Virkon Aquatic in 2007) is the only U.S. Environmental Protection Agency-registered disinfectant that is specifically labeled for use in aquaculture facilities to control a wide variety of viral, bacterial, and fungal pathogens (DuPont, 2010).

N-alkyl dimethyl benzyl ammonium chloride (Roccal-D Plus) is a veterinary disinfectant, but is not specifically labeled for use in fish. Sodium hypochlorite (Clorox), benzyl-4-chlorophenol/ phenylphenol (Lysol), ethyl alcohol, and glutaraldehyde are not specifically recommended for food fish aquaculture. Ultimately, the decision of which disinfectant is best for a particular aquatic situation depends on such factors as efficacy, volume required, cost, toxicity, and potential effluent concerns (Mainous and Smith, 2005). Some disinfectants may only be effective at excessively high concentrations or volumes; some may be too expensive. Other chemicals, such as formalin, glutaraldehyde, and chlorine, have been associated with human health risks, including respiratory illnesses and irritation of the skin and mucous membranes. In addition, disposal of formalin or glutaraldehyde through a municipal sewer system may be restricted. Phenolic compounds are also hazardous to animals and humans and leave a residue that may require thorough rinsing before the items or systems can be used (Rutala *et al*., 2008).

CONCLUSION

Disinfection is an important part of biosecurity to prevent disease outbreaks. Proper disinfection can be expected to be less expensive than economic cost due to antimicrobial treatment of an infected population, or loss of part or all of that population due to disease outbreak.

The study revealed that Biosentry Iodine, Aldekol des- Gda and Biosentry® 904™ were very effective against *Vibrio vulnificus* after 1 min contact time. Verkon-S, Aldekol des- Gda and TH4 were very effective against *Aeromonas hydrophila* after 1 min contact time. However, Verkon-S, Aldekol des- Gda , TH4, Peraclen and Biosentry® 904™ were effective disinfectants, as each reduced or eliminated the number of detectable organisms within 5 min of contact time with the exception of Biosentry® Iodine™

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Article's Citation:

Ismail E, Kaoud HA, Hamouda M and Abu-Ela N (2015). Bactericidal effectiveness of some commercial disinfectants against *Aeromonas hydrophila* and *Vibrio vulnificus*. *Ew J Microb Res* 1(1): 9-15.