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Study of Some Disinfectants Efficacy on *Aeromonas Hydrophila* Recovered from Local Animal and Water Sources

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Abstract: *A total of 145clinical and environmental sampleswere collected from(35pig, 30goat, 25sheep,40 rabbit and15drinking water sources) either apparently healthy or showed signs of diarrhea. The samples were cultured onto enriched and specific media (nutrient, blood and MacConkey agar and Aeromonas agar base with ampicillin) and identified biochemically. Aeromonas hydrophila isolated from apparent healthy, diarrheic animals and water sources with incidence of 24.2%, 25.6%, and 66.6% respectively. The impact of certain disinfectants; chlorine, formalin and virkon`S wasfulfilled using the minimum inhibitory concentration (MIC) method. The chlorine disinfectant was checkedat concentrations; 200, 1000, 5000 and 25000part per million in various times:5,10,15,30 minutes for each concentration. It was found that chlorine induced complete destruction at 200 ppm and 1000 ppm after 15 and 30 minutes respectively. Higher concentrations, 5000 ppm and 25000ppm managed to eliminate the microorganism completely after 5 minutes only. The efficiency of formalin was performed at concentrations: 1, 3, 5 and 7% for 5, 10, 15 and 30 minutes for each concentration. It was observed that 5% formalin could destroy the microorganism after 10 minutes while 7% formalin gave complete removal after 5 minutes. Finally, virkon`S was screened at concentrations: 0.01, 0.1 and 1% for 5, 10, 15 and 30 minutes and showed that it could suppress the growth completely at concentration 10 g/liter (1%) after 5 minutes exposure only. The phenol coefficient of virkon was the highest. The trial data deduced that virkon`S was the most efficient disinfectant against Aeromonas hydrophila isolates.*
Key Words*:Aeromonas hydrophila- animal- water- verkon S- disinfectant*

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I. Introduction

Aeromonas species are vastly distributed in aquatic environment[1].*A. hydrophila* comprises a public health concern;substantial humanpathogens causing primary and secondary septicemia in immune-compromised individuals, serious wound infections in healthy individuals, gastrointestinal and extra gastrointestinal illness[2,3].*A. hydrophila*has also been isolated from a wide range of sea foods, meat and dairy products[4].Animals appear to hold these organisms with or without clinical signs. Diarrhea is thecommon illness. They have been isolated from 0.5% to 62.5%(mean = 8%) meat animals (i.e. cattle, buffalo, camel, sheep, goat and pig) and from 0.0% to 29% (mean = 14%) poultry and other birds. They havealso been isolated from domestic cats, dogsandbound birds. Appearance of *A. hydrophila*in animals may be correlated to existence of the organisms in theirfood and drinking water [5].Since these microorganisms significantly contribute to human and animal illness and death. Different ways have been mentioned to either eradicatethe pathogenic bacteria totally or just diminish the number of viable cells.The successful extirpation of these pathogens with antibiotics has been complicated by the development of highly resistant strains as well as the release of new virulent pathogens[6].

Disinfectants constitute a group of non- antibiotic antimicrobial agents of different compositions "majority chemical", which destruct or prohibitthe growth of microorganisms and can be sporostatic. Disinfectants may inactivate cells in a different ways involving damage of cell wall or cytoplasmic membrane, electron transport obtrusion and the coagulation of nucleic acids and proteins. Chemical disinfection is convenient for the decontamination of liquid wastes, as well as solid infectious wastes and considered more efficient in diminishing the number of bacteria than the cleaning with water and soap[7].They could be organizedinto three categories [1]High- level disinfectants (destruct, fungi, viruses,vegetative bacteria, and some bacterial spores. [2]Intermediate-level disinfectants (eliminate tubercles bacilli,vegetative bacterial cells, and the majority of fungi). [3] Low-level disinfectants (act only on fungi and able to destroy bacteria in vegetative form)[8].

The random use of antibiotics is prospectively capable of producing a higher infection incidence with drug resistant bacteria such as *A. hydrophila*.This biofilm forming microorganism recorded as problematic

organism in exhibiting resistance not only to antibiotic but also to disinfectants[9].Otherwise, using the convenient disinfectant looked as a primary part of infection control practices.The utilizing of liquiddisinfectantsin animal laboratories is admitted as sharing with the keeping of good hygienic measures[10].

The goals of our study were todetermine the incidence of*A. hydrophila*among livestock animalsin some Egyptian localities.The efficacy of certain commercial disinfectants against isolated*A. hydrophila*was assessed.The minimum inhibitory concentration, the time exposure were checked to obtain complete destruction of the microorganism.

II. Material And Methods

Samples: A total 145 of samples were collected as fecal swabs(130), from apparently healthy or diarrheic back yardreared animals (35 pig, 30 goat, 25 sheep,40 rabbit) and drinking water sources samples (15) in Giza and Helwan districts.

The animal sampleswere labeled with the primary essential identifications (animal number, case history). Water samples were taken under aseptic condition and centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and 1 ml was taken from sediment and inoculated onto 9 ml of sterile nutrient broth then incubated at 37 C for 24 hours. A loopful from incubated tubes was cultured onto different culture media.

Bacteriological examination:All samples were inoculated into the following media: nutrient agar, blood agar, MacConkey agar and *Aeromonas* agar base with ampicillin and were incubated aerobically at 37ºC for 24-48 hours. The developed colonies were picked up and sub-cultured for purification. The pure colonies were identified morphologically and biochemically by API 20E system (Bio Merieux) for identification of *A. hydrophila* after 24 hrs incubation was performed according to manufacturer's instruction to detect the biochemical profile of the isolated organisms[11].

Efficiency of certain disinfectants: the effect of tested disinfectants; chlorine, formalin and Virkon`S on isolated *A. hydrophila* was carried out by using minimum inhibitory concentration method (MIC) according to Mazzola *et al.*[12].

Table (1): Disinfectants and theirconcentrations

Disinfectants	Dilution	Concentration
Chlorine (4.6% sodiumhypochlorite)	1 part to 250 parts 1 part to 50 parts 1 part to 10 parts 1 part to 2 part	0.02% (200 ppm) 0.1% (1000ppm) 0.5% (5000 ppm) 2.5 % (25000 ppm)
Formalin (37%formaldehyde)	10 part to 1000 parts 30 parts to 1000 parts 50 parts to 1000 parts 70 parts to 1000 parts	1% 3% 5% 7%
Virkon`S (20.4% potassium peroxy-monosulphate ,1.5% sodium chloride)	0.1 g/l 1.0 g/l 10 g/l	0.01% 0.1% 1.0%

Preparation of culture suspension: The isolated *A. hydrophila*was purified on nutrient agar and incubated for 24 hours at 37º C, then transferred in a test tube containing sterile saline to make matching with MacFerland tube No 1, containing approximately ($3x10^8$ C.F.U./ml).

Test performance:

Determination of minimum inhibitory concentration (MIC) for every chemical agent was developed, through the classic method of successive dilution. In twelve numbered screw tubes (10 x 100 mm), 1 ml of trypticase soy broth (TSB) medium was distributed for every tube, except for the tube number 1. Tubes were submitted to autoclave under constant pressure and temperature of 121 °C. For the first and the second tubes of the series, 1 ml of tested sanitizing agent was added; tube 2 was stirred and 1 ml was withdrawn and transferred to tube 3. This successive transference was repeated until tube 11. It was added to all flasks, except for flask number 11, 0.1 ml of inoculation (tested microorganism) at known concentration. Incubation at optimal temperature was developed for 24 and 48 hours. After this period, the reading was recorded; the MIC is the concentration of the higher dilution tube in which the absence of bacterial growth occurred. Tubes 11 and 12 are positive (TSB + inoculation) and negative (TSB + antimicrobial) controls[13].

Determination of decimal reduction time of chemical agents used for disinfectant purposes:

A drop of disinfectant bacterial mixture from a previously serial dilution was applied over the surface of standard plate count agar at the time intervals 5, 10, 15, and 30 minutes from original zero time. Dilution of the chemical agent to the appropriate bactericidal concentration must be effected with clean water. Meanwhile, direct contact with dirty materials should be avoided, the presence of which cause gradual loss of strength and

become a vehicle of contamination to other surfaces. Solutions of chemical agents should be kept in closed containers, well protected from air contaminants and provided with a facility which releases a constant required amount[14].

Determination of phenol coefficient (Rideal-walker coefficient) for tested disinfectant.

Series dilutions of phenol and the examined disinfectants are prepared. A standard amount of *A. hydrophila* strain ($3x10^8$) were added to each dilution at 5 time intervals, samples were inoculated on a growth medium which was incubated for 24-48 days at 37º C. Then the growth was examined. The highest dilution that kills the bacteria after 10 minutes exposure but not 5 minutes is used to calculate the phenol coefficient[15].

The phenol coefficient was calculated as follow:

Phenol coefficient =

Thehighest dilution of virkon after 10 minutes exposure

———————————————————

The highest dilution of phenol after 10 minutes exposure

III. Results

The cultural properties of *A.hydrophila*exhibited smooth, convex, rounded colonies on nutrient agar,β-hemolytic white to grey colonies on blood agar, non-lactose fermenterpale colonies on MacConkey agarand dark green, opaque colonies on selective media. All isolates were gram negative and rod shaped. As*Aeromonas* spp.could be differentiated from *Enterobacteriaceae* by the positivity of oxidase testand further biochemical identification of *A. hydrophila* using API confirmed by production of acid from fermentation of sucrose, L-arabinose, and urocanic acid but not from salicin[16].

*A. hydrophila*was isolated from (22/91) clinical apparent healthy animal cases with incidence of 24.2% while the microorganism was recovered from (10/39) diarrheic animals with incidence of 25.6%. The highest isolation was obtained from water samples (10/15) 66.6%.

Data shown in table (2, and 3) revealed the detail about the *A. hydrophila* animal isolatesincidences.

Table (2): The rate of isolation of *A. hydrophila* from apparent healthy animals.

Samples	No. of examined samples	No. of Positive samples	%
Sheep	16	6	37.5
Goat	23	8	34.8
Pig	24	3	12.5
Rabbit	28	5	17.8
Total	91	22	24.2

Table (3): The rate of isolation of *A. hydrophila* from diarrheic animals.

Samples	No. of examined samples	No. of Positive samples	%
Sheep	9	1	11.1
Goat	7	1	14.3
Pig	11	5	45.4
Rabbit	12	3	25
Total	39	10	25.6

The obtained data exhibited near incidence ratios between the apparent healthy and diarrheic animals.

The influence of certain disinfectants against *A. hydrophila* isolated from various sources was illustrated. The data in table (4) revealed that*A. hydrophila* which isolated from water sources were relatively resistant to the action of chlorine concentration of 200 ppm at 15 minutes exposure.Whilechlorine concentration of 1000 ppm prohibitedboth animal and water origin isolates after 15 minutes. The highest concentration of this disinfectant (5000 ppm and 25000ppm) killed the organism completelyat 5 minutes display.

Table (4): Bactericidal action of chlorine on*A. hydrophila* isolates.

Concentration of chlorine	Number of microorganisms	Exposure time per minute			
		5	10	15	30
200 ppm (0.02%)	$3 \times 10^{8/}$ml	+	+	+	-
1000 ppm (0.1%)	3×10^{8}ml	+	+	-	-
5000 ppm (0.5%)	3×10^{8}/ml	-	-	-	-
25000ppm (2.5%)	3×10^{8}/ml	-	-	-	-

As shown in table (5), *A. hydrophila* of assorted origins could resist 1% and 3% formalin for 15 minutes exposure while, after 30 minutes complete inhibition of growth happened. It was noticed that 5% formalin had a marked killing effect on the growth after 5 minutes. Concentration of 7% has powerful bactericidal effect on the examined organism.

Table (5): Bactericidal action of formalin solution on *A. hydrophila* isolated isolates.

Concentration of formalin	Number of microorganisms	Exposure time per minute			
		5	10	15	30
1%	3 x 10^8/ml	+	+	+	-
3%	3 x 10^8/ml	+	+	+	-
5%	3 x 10^8/ml	+	-	-	-
7%	3 x 10^8/ml	-	-	-	-

Theeffect of the various concentrations of virkon`S on isolated*A. hydrophila*was shown in (table 6). Itappeared that all *A. hydrophila* isolates could resist the effect of 0.01% concentration virkon`S at all examined times. Using concentration of 0.1% could eradicate the microorganism after 30 minutes exposure. On the other hand virkon`S 1% concentration was capableto kill all isolates of *A. hydrophila* before 5 minutes.

Table (6): Bactericidal action of Virkon`S on *A. hydrophila* isolated strains.

Concentration of Virkon`S	Number of microorganisms	Exposure time per minute			
		5	10	15	30
(0.01%)	3 x 10^8/ml	+	+	+	+
(0.1%)	3 x 10^8/ml	+	+	+	-
(1%)	3 x 10^8/ml	-	-	-	-

The phenol coefficient of previous disinfectants was determinedand the results showed that the highest one was Virkon`S≈1.05, followed by formalin≈ 0.9 then the lowest one was chlorine ≈ 0.5.

IV. Discussion

Aeromonas hydrophila, is Gram-negative bacterium commonly inhabitant in aquatic environments, and known as important fish pathogen[17]. Recently, this microorganism has increasingly been implicated in human and livestock animal diseases. The severity of diseases varied from mild diarrhea to fatal septicemia and necrotizing inflammation in many internal organs[18]. Furthermore, the public health concern of the microorganism is increased as the ability of pathogen to contaminate meat and milk products[19].

A. hydrophila was isolated from apparent healthy sheep (6/16) 37.5% and from diarrheic cases (1/9) 11.1% these incidences were coincided with (10 - 47.6%) that obtained by Moses[20](14.0%) Melas *et al.*[21]and (10%) Ceylan*et al.*[22].

The results of *A. hydrophila* isolation from goat were 34.8% in apparently health and 14.3% in diarrheic cases. These results similar to that obtained by Moses[20] (17.6% - 39.3%) but far from those of Sharma and Kumar[23] (2.5 %) and Gowda *et al.*[24] (52-60 %).

Regarding,the pig results which varied from 12.5% in apparent health to 45.4% in diarrheic cases. These results agreed with (45.1 %) that obtained by Igbinosa *et al.*[25], but twice the results of Gowda *et al.*[24](22-30%) and ten times more than the results revealed by Evangelopoulou *et al.*[26] (4.6%).

The result of *A. hydrophila* isolation in apparent healthy rabbit was 17.8% and in diarrheic rabbits was 25%. The result of apparent healthy cases close to those of Gowda *et al.*[24] (20%) but the result of diarrheic cases was greatly lower than data obtained in the same study (88.1%). Another study revealed *A. hydrophila* isolation from rabbit by incidence of 35.3% [27].

Finally, *A. hydrophila* was isolated from drinking water sources by the incidence of 66.6%, Fernández *et al.*[28], Scoaris[29], and Egorov *et al.*[30]mentioned the high isolation incidence of *A. hydrophila* from water sources.

Microorganisms differ greatly in their resistance to disinfectants; Gram negative bacteria are more resistant due to possessing an outer membrane that serves as a barrier to the uptake of disinfectants in addition tothe ability of some microorganisms to create resistant biofilm[31]**.**

Because of the ability of *A. hydrophila* tosurvive in unfavorable environmental conditions and its high resistanceto antibacterial agents, besides the public health concern. This microorganismcontinues to be asubstantial pathogen in ponds, farms and acquired infections[32].

So there is a great need to obtain the suitable efficient disinfectant against *A. hydrophila* and our study was designed to examine certain disinfectants which traditionally used.

The given results in table (4) exhibited that the utilizing of chlorine concentration of 200 ppmof (0.02%) and 1000 ppm (0.1%) failed to clear*A. hydrophila* before 30 and 15 minutes respectively. While higher concentrations as 5000 ppm (0.5%) and 25000 (2.5%) had a complete bactericidal influence after 5 minutes. These data were harmonized with that stated by Martínez-Hernández *et al*.[33]who demonstrated the requirement of a high chlorine concentration and long time for *A. hydrophila* killing.

These results are nearly confirmed byRutala and Weber[34]who declared that chlorine is ideally diluted 1:50 (1000 ppm) for superficial disinfection while using 5000 ppm to clean up blood spills. Chlorine has been used in water treatment, but should not exceed 6-10 ppmin drinking water. Chlorine is deemed an intermediate-level disinfectant, have a broad spectrum of antimicrobial activity, do not leave toxic remains, inexpensive and fast acting. On the other hand it has corrosive effect, and post health risks. So optimizing chlorine implementation orlooking for alternatives sanitizing agents isessential to minimize the dispense of chlorinated residues to the environment[35].

Concerning the impact of formalin as a bactericidal agent,the afforded resultsshowed that 1% and 3% formalin concentrations were considered weakly effective against *A. hydrophila* till 30 minutes exposure. On the contrarythe using 5% formalin, complete elimination ofthe organismwas occurred in5 minutes exposure, and then the increasing to concentration of 7% solution destroyed all tested isolates at once. Marcia *et al*.[36]mentioned that the usingof formalin was effective when tested againstGram negative bacteriaand it is recommended as instrument disinfectant. Formalin is a high-level disinfectant at variable concentrations eradicates a wide range of microorganisms. The aqueous solutionis able to kill bacteria, tubercles bacilli, fungi, viruses and spores. It can interact with RNA and protein in vivo. So the adverse impact of formalin is attributed to its genotoxic and carcinogenic property at long term display[37]. Moreover, the exposure to low level inhalation or skin touch can predispose to asthma like respiratory illness and skin irritation such as dermatitis and itching. So that, workers should restrict their direct contact with formalin and that interfere with its role in disinfection and sterilization approach[35].

The effect of the various concentrations of virkon`S on isolated*A. hydrophila*was shown in (table 6). It appeared that all *A. hydrophila* isolates could resist the effect of 0.01% concentration virkon`S at all examined times. Using concentration of 0.1% could eradicate the microorganism after 30 minutes exposure. On the other hand the obtained results displayed that the efficacy of virkon`S at 1% was very high; it could eliminate *A. hydrophila* completely in only 5 minutes. This was in correspondence with that reported by Chereen *et al*.[38]who pointed that virkon`S is effective in 5-30 minutes resulting in no growth after cleaning. Virkon`S is a potentwidespectrum multipurpose disinfectant either under clean or dirty circumstances. Virkon`s would not have irritant or toxic effect on animals and human[39].

The efficiency of a disinfectant or antiseptic can be evaluated in a number of methods. A common way is the accounting of phenol coefficient which may be determined as killing potency of antimicrobial agent against the tested microorganism parallel to that of phenol[40].

Our results declared that the highest phenol coefficient was belonged to virkon `S was ≈1.05, followed by formalin ≈ 0.9 then the lowest one was chlorine ≈ 0.5suggesting the high efficiency of virkon`S in compared with the other two disinfectant[41,42].

V. Conclusion

Aeromonas hydrophila is emerging pathogen which should have to pay attention to its existing and isolation from house reared animals due to its ability to transmit to human resulting in bad consequences.So elimination of this microorganismis a great objective seeking about a rapid, powerful,non- toxic,and safe to environment. The present study tested the activity of three common used disinfectants versus *A. hydrophila*isolates of local animal and drinking water sources.The results elucidated that the efficacy of virkon`Swas higher than chlorine and formalin regarding to its advantages as safe to animal and environment.

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