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Combined Effect Of Disinfectant And Phage On The Survivality Of S. Typhimurium And Its Biofilm Phenotype

Mudit Chandra*, Sunita Thakur, Satish S Chougule, Deepti Narang, Gurpreet Kaur and N S Sharma

Department of Veterinary Microbiology, College of Veterinary Science, GADVASU, Ludhiana, Punjab, -141004, India

Abstract

In the present study bacteria and biofilm phenotypes were treated with various disinfectants (Phenol 5%, Ethanol 70%, Hydrogen peroxide 30%, and Iodine 5%) to observe the effect of disinfectants on bacteria and biofilm. It was observed that none of the disinfectants tested were effective in removing biofilm completely. Finally bacteria and biofilm phenotypes were incubated with phage and 400 and 800 ppm concentrations of the disinfectants (phenol, iodine, sodium hypochlorite and benzalkonium) together, revealed that it is possible to eliminate biofilm by combining phenol, iodine or sodium hypochlorite along with phage whereas benzalkonium was effective in eliminating biofilm with or without addition of phage indicating its usefulness.

Key words: Salmonella, biofilm, Phage, phenol, Iodine, benzalkonium

Introduction

Salmonella is an important member of the family Enterobacteriaceae that causes bacterial food poisoning worldwide (Mead et al. 1999; Stark et al. 2009). It has two species enterica and bongori. S. enterica has been further sub divided into six subspecies viz., enterica, salamae, arizonae, diarizonae, houtenae and indica. More than 2500 serovars of Salmonella enterica subspp enterica are responsible for a variety of diseases in animals and human being. Among these Salmonella Enteritidis (S. Enteritidis) and S. Typhimurium are the most frequently identified Salmonella causing diseases in humans and animals.

Bacteriophages are the viruses that infect bacteria and can either kill or integrate its genome with that of its host. After the initial success of phage as a therapeutic agent its use was mostly shelved mainly due to the advent of antibiotics in the west, but, due to the development of multiple drug resistant bacteria renewed interest has been instigated into it. It has been reported that wherever bacteria thrive it is possible to find phages (Ashelford et al. 2003; Dabrowska et al. 2005 Furuse 1987; Merril 1974) and bacteriophage cocktail when administered has delivered therapeutic results particularly for the control of Salmonella (Andreatti Filho et al. 2007), Escherichia coli (Huff et al. 2002) and Campylobacter (Goode et al. 2003). However, complete elucidation of prophylactic and therapeutic potential of bacteriophages is still incomplete and needs further studies.

Biofilm is a community of bacteria living under an organized system (Davies 2003) where bacteria are provided a very conducive environment for its establishment and survival (Costerton et al. 1994). Biofilm has been observed for both pathogenic and nonpathogenic bacteria (Deibel and Schoeni 2003). It provides protection and exchange of nutrients; but remains a continuous source of contamination. It has been observed that biofilm forming capacity is widespread among natural isolates of S. Enteritidis and S. Typhimurium (Solano et al. 2002; Zogaj et al. 2001). Salmonella biofilms develops comfortably when it is exposed to various stresses (chemical, desiccation/starvation) under which its normal phenotype will not withstand (Szomolay et al. 2005). Recently it has been shown that different bacteria undergo transitions from the planktonic mode to the biofilm mode of growth and that these transitions include the timed expression of different sets of genes and proteins (Sauer and Camper 2001). Lot of research is undergoing to eliminate biofilm phenotypes

*Corresponding author mailing address: Department of Veterinary Microbiology, College of Veterinary Science, GADVASU, Ludhiana, Punjab, India, Tel:+91-1612414033, Fax: +91-161-2400822, Email: drmuditchandra@rediffmail.com

using various disinfectants and antimicrobial and it has also been observed that bacteriophages eliminate biofilms of P. fluorescens (Sillankorva et al. 2004) and L. monocytogenes (Hibma et al. 1997).

Keeping in mind the above facts, the present study was conducted to evaluate effect of phages in combination with various disinfectants for controlling biofilm of S. Typhimurium.

Materials and Methods

Bacterial Isolate. Salmonella Typhimurium available in the Department of Veterinary Microbiology, Ludhiana, India was revived and tested biochemically for its purity. Later it after confirmation it was maintained on nutrient agar slant at 4oC until the completion of the study.

Isolation of Phage against Salmonella Typhimurium. Phages against S. Typhimurium were isolated as per the protocol (Chandra et al. 2011). In brief, equal volume of the sewage sample was dispensed in 2 X NZCYM broth (New Zealand Casamino Yeast Medium, HiMedia, Mumbai) and incubated for 18 h at 37 oC. From this 10 ml was aspirated and centrifuged at 8000 X g for 10 minutes and the supernatant was passed through 0.45 micron filter (Axiva, Kolkata) and later with 0.22 micron filter (Axiva, Kolkata) and was designated as Bacteria free filtrate (BFF). Later, BFF and log phase bacterial growth (6h growth) were suspended in NZCYM semisolid broth at 40-45oC and instantly poured onto NZCYM Agar plates. The plates were incubated for 18h at 37oC to observe for plaque. The plaques were streaked in grid onto fresh NZCYM agar plate on which semisolid NZCYM medium and bacteria were poured. The clearing along the streaks confirmed the presence of phage. The phage was stored in SM (Suspension Medium) buffer and kept at 4oC.

Formation of Biofilm. Biofilm was formed in Trypticase Soy Broth (TSB) by adding 1% chitin flakes (W/V, Sigma), sterilized glass slides to 12 h growth of Salmonella in a large petri plate. It was incubated for 7 days at 37oC and biofilm phenotypes were scrapped from the plates.

Effect of exposure of disinfectants on bacteria and biofilm. The experiment was performed in 96 well polystyrene plates (GenAxy, India). In brief 0.1ml of bacteria (approximately 109CFU/ml) and biofilm (approximately 109CFU/ml) phenotypes were subjected to various concentrations (25, 50, 100, 200, 400 and 800 ppm) of different disinfectants (Phenol 5%, Ethanol 70%, Hydrogen peroxide 30%, and Iodine 5%). They were incubated and Optical Density (OD) at 570 nm was recorded at various time intervals (0, 5, 15, 30 and 60 min).

Combined effect of phages and various disinfectants on the bacteria and biofilm. The experiment was performed in 96 well polystyrene plates (GenAxy, India). The experiment was performed by growing the bacterium and the biofilm phenotype for 12 h in TSB. Later equal quantity of bacterium 0.1 ml of (approximately 109 CFU/ml) or biofilm (approximately 109 CFU/ml) phenotype were incubated with 0.1 ml of phage (approximately 2.5*1010PFU/ml) up to 2 h to observe for the effect of phage on the bacterium or biofilm. Also 0.1 ml of (approximately 109 CFU/ml) bacteria or biofilm (approximately 109 CFU/ml) phenotype were incubated with 0.1 ml of phage (approximately 2.5*1010PFU/ml) and 0.1 ml of disinfectants (Phenol, Iodine, Sodium Hypochlorite and Benzalkonium) up to 2 h to observe for the effect of phage on the bacterium or biofilm when incubated simultaneously with a disinfectant.

Statistical analysis. The data were analyzed by one way ANOVA using General Linear Model procedure of SPSS (9.0). Means were compared using Tukey's test.

Results

Isolation of Phages against Salmonella Typhimurium. A total of 10 sewage samples were evaluated for the presence of phages against S. Typhimurium and two phages (P1 and P2) were isolated. These phages were partially characterized against various pH and temperature range to observe their stability. Among both P2 phage was selected in this study keeping in view its stability between pH 4-10 and resistance to the variation in temperature 50 oC (data not shown).

Formation of Biofilm. After 7 days of incubation in TSB with chitin it was observed that S. Typhimurium formed biofilm successfully.

Effect of exposure of disinfectants on bacteria. Exposure of various disinfectants at various concentrations revealed that at various time intervals (0, 5, 15, 30 and 60 min) there was significant variation on the bacterial concentration depending upon the exposure time ($F=3.55$; $P=0.009$) as there was significant variation between the initial and final ($P=0.013$) exposure time.

When individual disinfectants effect was compared with one another it revealed that there was significant variation ($F=6.28$; $P= 0.0005$) between the effect of various disinfectants and all the disinfectants were having almost similar (non-significant) activity on bacteria at various time period exposure ($F=0.61$; $P=0.821$) except Iodine that was effective at 800 ppm (Table 1).

Effect of exposure of disinfectants on biofilm phenotypes. Exposure of various disinfectants revealed that at various time intervals there was significant variation on the biofilm concentration depending upon the exposure time ($F=2.91$; $P=0.024$) as there was significant variation between the initial and final exposure time ($P=0.033$).

When individual disinfectants effect was compared with one another it revealed that there was significant variation ($F=4.86$; $P=0.003$) however, all the disinfectants were having almost similar (non-significant) activity on biofilm

Table 1 Effect of disinfectants on biofilm and bacteria

Time (min)	25ppm		50 ppm		100 ppm		200 ppm		400 ppm		800 ppm	
	Bio	**Bac**	**Bio**	**Bac**	**Bio**	**Bac**	**Bio**	**Bac**	**Bio**	**Bac**	**Bio**	**Bac**
Phenol												
0	0.669	0.141	0.651	0.14	0.638	0.139	0.632	0.135	0.631	0.132	0.619	0.13
5	0.668	0.14	0.632	0.139	0.632	0.139	0.631	0.131	0.63	0.131	0.585	0.13
15	0.66	0.14	0.63	0.138	0.63	0.138	0.63	0.138	0.627	0.13	0.577	0.129
30	0.66	0.14	0.63	0.138	0.63	0.138	0.628	0.13	0.62	0.13	0.574	0.128
1 h	0.66	0.14	0.63	0.131	0.63	0.131	0.618	0.129	0.614	0.128	0.56	0.127
Ethanol												
0	0.707	0.155	0.7	0.145	0.694	0.145	0.668	0.144	0.599	0.139	0.527	0.126
5	0.681	0.155	0.676	0.144	0.671	0.144	0.653	0.144	0.581	0.139	0.524	0.122
15	0.653	0.153	0.653	0.144	0.627	0.143	0.619	0.14	0.519	0.13	0.51	0.12
30	0.651	0.152	0.65	0.143	0.627	0.143	0.617	0.14	0.509	0.13	0.5	0.12
1 h	0.65	0.15	0.648	0.143	0.617	0.141	0.61	0.138	0.5	0.128	0.5	0.12
Hydrogen peroxide												
0	0.679	0.164	0.678	0.158	0.678	0.155	0.667	0.145	0.634	0.145	0.622	0.128
5	0.677	0.161	0.67	0.157	0.664	0.155	0.66	0.144	0.63	0.144	0.621	0.127
15	0.668	0.159	0.668	0.15	0.663	0.154	0.659	0.14	0.617	0.139	0.614	0.126
30	0.668	0.159	0.667	0.149	0.659	0.153	0.645	0.139	0.613	0.139	0.615	0.126
1 h	0.662	0.156	0.648	0.145	0.652	0.15	0.63	0.137	0.609	0.136	0.608	0.125
Iodine												
0	0.706	0.172	0.702	0.165	0.671	0.156	0.663	0.152	0.621	0.147	0.619	0.133
5	0.704	0.163	0.66	0.16	0.66	0.148	0.659	0.143	0.612	0.139	0.612	0.13
15	0.7	0.161	0.653	0.153	0.653	0.138	0.639	0.137	0.61	0.137	0.607	0.12
30	0.651	0.15	0.65	0.136	0.65	0.137	0.619	0.134	0.608	0.13	0.603	0.13
1 h	0.648	0.146	0.649	0.138	0.644	0.135	0.628	0.129	0.605	0.126	0.601	0.119

Table 2 Effect of disinfectants in percentage on the biofilm and bacteria along with phage

Phenol										
Time (h)	**Bi+P**	**Bi+400 P**	**Bi+800 P**	**Bi+P+400P**	**Bi+P+800P**	**B+P**	**B+400P**	**B+800P**	**B+P+400P**	**B+P+800P**
0	100	100	100	100	100	100	100	100	100	100
0.5	120.3	108.0	178.3	63.2	39.1	52.0	32.2	84.0	64.7	81.6
1	167.8	114.9	378.3	38.6	51.7	44.0	29.9	48.0	29.4	49.4
2	194.9	154.0	482.6	29.8	54.0	36.0	24.1	32.0	23.5	18.4
Iodine										
0	100	100	100	100	100	100	100	100	100	100
0.5	120.3	118.7	71.6	96.6	60.7	52.0	65.2	30.9	93.5	40.9
1	167.8	133.3	100.0	89.8	93.3	44.0	88.8	16.2	70.8	18.2
2	194.9	115.4	109.9	83.9	94.4	36.0	91.9	1.5	47.6	0.0
Sodium Hypochlorite										
0	100	100	100	100	100	100	100	100	100	100
0.5	120.3	41.8	83.6	48.3	56.3	52.0	60.2	37.5	23.1	11.1
1	167.8	52.1	26.4	41.6	0.8	44.0	38.6	33.0	7.7	0.0
2	194.9	52.1	23.6	38.8	0.0	36.0	19.3	8.0	0.0	0.0
Benzalkonium										
Time (h)	**Bi+P**	**Bi+25 B**	**Bi+50 B**	**Bi+P+25B**	**Bi+P+50B**	**B+P**	**B+25B**	**B+50B**	**B+P+25B**	**B+P+50B**
0	100	100.0	0	100.0	100.0	100	100.0	100	100.0	0
0.5	120.3	92.3	0	83.8	27.8	52.0	88.5	150	91.8	0
1	167.8	84.6	0	73.0	5.6	44.0	70.5	100	71.4	0
2	194.9	57.7	0	60.8	0	36.0	59.0	0	63.3	0

Bi: Biofilm B: Bacteria; P: Phage; 400P: 400ppm Phenol; 800P: 800ppm Phenol; 400I: 400ppm iodine; 800P: 800ppm iodine; 400N: 400ppm sodium hypochlorite; 800N: 800ppm sodium hypochlorite; 25B: 25ppm benzalkonium; 50B: 50ppm benzalkonium

Bio: Biofilm phenotypes; Bac: Bacteriaat various time period exposure (F=0.30; P=0.986) (Table 1).

Combined effect of phage and various disinfectants on bacteria. Phenol. When the effect of phenol at 400ppm and 800ppm was evaluated along with phage on the bacteria it was found that there was significant variation at various time of exposure (F=8.0; P= 0.003) indicting combined effect of phage and 800ppm phenol on bacteria.

Iodine. When the effect of iodine at 400ppm and 800ppm was evaluated along with phage it was found that there was significant variation at various time of exposure (F=3.74; P= 0.041) indicating that combined effect of phage and 800ppm of iodine significantly reduced bacteria.

Sodium Hypochlorite. When the effect of sodium hypochlorite at 400ppm and 800ppm was evaluated along with phage it was found that there was significant variation at various time of exposure (F=6.31; P=0.008). It was observed that, when phage was incubated along with the sodium hypochlorite at either 400 or 800 ppm it completely inhibited bacterium.

Benzalkonium. When the effect of benzalkonium at 400ppm and 800ppm was evaluated along with phage it was found that there was non-significant variation at various time of exposure (F=1.0; P=0.042) indicating its efficacy at the very start of the experiment. Moreover, it was observed that, when phage was incubated along with the benzalkonium at either 400 or 800 ppm it completely inhibited bacterium and so, it was evaluated on 50 and 25 ppm concentration and revealed that effect of benzalkonium alone was most prominent in reducing bacteria (Table 2).

Combined effect of phages and various disinfectants on the biofilm phenotype. Phenol. At 400ppm and 800ppm phenol with phage on the biofilm it was found that there was reduction of biofilm at 400ppm. There was significant variation (F=4.369037; P=0.020748) indicating effect of combined effect of phage and phenol on the ability of either phenol or phage to act on the biofilm and decreasing its concentration.

Iodine. At 400ppm and 800ppm iodine with phage on the biofilm it was found that there was significant variation (F=17.21; P=0.00) indicating little combined effect of phage and iodine on the biofilm.

Sodium Hypochlorite. At 400ppm and 800ppm phage on the biofilm it was found that there was non-significant (F=0.31; P=0.81) effect at various time of exposure. However, between different groups there was again non-significant variation (F=1.01; P=0.436) indicating no effect of combined use of phage and sodium hypochlorite on the ability of either sodium hypochlorite or phage to act on the biofilm and decreasing its concentration.

Benzalkonium. At 400ppm and 800ppm with phage on the biofilm it was found that there was non-significant (F=1.0; P=0.426) effect at various time of exposure. When the effect of benzalkonium was evaluated at 25 ppm and 50 ppm concentration and it revealed that with or without phage at 50ppm biofilm was reduced completely (Table 2).

Discussion

Salmonellosis is a very important bacterial disease affecting both human beings as well as animals. Salmonella is present both in the host as well as in the environment (Wray and Wray 2002).

After 7 days of incubation in the TSB along with chitin it was observed that S. Typhimurium formed biofilm successfully. The formation of biofilm by Salmonella and other bacteria has been successfully reported by many earlier workers (Esteves et al. 2005; Gough and Dodd 1998; Joseph et al. 2001; Stepanovic et al. 2004). Murphy and Kirkham (2002) estimated that 99.9 % of the bacteria in nature attach to a surface in the form of biofilm. It has also been observed that formation of biofilm plays a predominant role in the establishment and pathogenesis of numerous bacterial species and thus may be essential for an organism to express its pathogenic potential (Costerton et al. 1999; Watnick and Kolter 2000).

Disinfectants are chemicals used to inhibit or prevent the growth of microbes on inanimate objects (Rossoni and Gaylarde 2000). The focus on safer foods and longer shelf-life has led to more frequent use of chemical disinfectants (Langsrud et al. 2003). In the present study the efficacy of four disinfectants viz, phenol, ethanol, hydrogen peroxide and iodine when evaluated on biofilm and bacteria revealed that most of them didn't affect biofilm at the concentrations tested so in the subsequent study ethanol and hydrogen peroxide were omitted and instead sodium hypochlorite and benzalkonium were included in the study. Bacteriophages are viruses that infect bacteria and may provide a natural, highly specific, non-toxic, feasible approach for controlling several microorganisms involved in biofilm formation (Kudva et al. 1999). The technology for this has not yet been successfully developed and relatively little information is available on the action of bacteriophages on biofilms (Hughes et al. 1998; Sillankorva et al. 2004; Sutherland et al. 2004) prompted us to investigate role of bacteriophage in killing biofilm. S. Typhimurium biofilm phenotypes were thus treated with bacteriophage alone and along with 400 and 800ppm of various disinfectants. The results suggested that along with bacteriophage at 400 and 800 ppm phenol and sodium hypochlorite were very good in eliminating biofilm whereas iodine was moderate and benzalkonium had no significance of adding phage as it was able to kill biofilm at very low ppm i.e. 50.

We found that bacterium was not cleared when treated with the phage alone is in alignment of the earlier findings (Ashelford et al. 2003; Furuse 1987; Neve et al. 1994) where they stated that despite phages clearly outnumbering bacteria in essentially all studied environments in biosphere they are unable to eliminate all the bacterium, we found that phages didn't reduced the bacterium significantly.

The effect of benzalkonium on S. Enteritidis biofilm revealed that at even 50 ppm it completely killed the biofilm within minutes of interaction. Our observations are

similar to the observations recorded by Mangalappalli-Illathu et al. (2008) who examined the effect of different concentrations of benzalkonium on biofilms reported rapid erosion and loss of biomass when biofilms grown for 48 h were continuously exposed to 10 µg/ml benzalkonium.

Conclusions

From the study it could be concluded that it is possible to eliminate biofilm by combining phenol, iodine or sodium hypochlorite with phage. Also, it is possible to eliminate Salmonella biofilm by using 50ppm of benzalkonium.

Conflict of Interests

The authors declare that there is no conflict of interest.

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