In vitro Comparative Activity of Chloramphenicol, Virulent Bacteriophages and the combination of both on Salmonella Species

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ABSTRACT

The Optical Densities (OD) of both Salmonella species (Ss) and Salmonella Pollurum (Sp) growth after 24 hrs. Infection by phages remained low (SsDW-0.2 ± 0.11; SsKW-0.37 ± 0.15 and SsR-0.19 ± 0.11) while the uninfected control Ss increased to 0.64±0.055. The decrease in OD600 of SsDW, SsR and SsKW were not significantly different from each other at p≤0.05. The difference in OD600 of SpDW (0.25±0.02309 to 0.36±0.03), SpKW (0.2767 ±0.006667 to 0.4667±0.06119) and SpR (0.2467 ±0.02028 to 0.2033±0.08413) were significantly different from each other at p≤0.05 except SpDW and 2SpKW which are not significantly different from each other but lower than the uninfected culture (Sp-0.62±0.02887). The final absorbance of the SsDW (0.2 ±0.11), SsC (0.17 ±0.05) and the combination SsDWC (0.37 ± 0.2) decreased significantly (p≤0.05) compared to that of Ss (0.64 ± 0.055). The activity of DW and DWC were not significantly different from each other. There were significant differences at p≤0.05 between the reductions due to Chloramphenicol (SsC-0.23 ± 0.0033 to 0.17 ± 0.05) and SsKW (0.23 ± 0.015 to 0.37 ± 0.15) compared to SsKWC (0.24 ± 0.01 to 0.14 ± 0.1). Between KW and C, the actions are not significantly different. There is no significant difference between the change in optical densities of SsR (0.25 ± 0.012 to 0.19 ± 0.11) and SsC (0.23 ± 0.0033 to 0.17 ± 0.05) and SsRC (0.23 ± 0.0033 to 0.17 ± 0.13).The optical densities of Sp increased significantly, being the control to 0.62 ± 0.02887 while SpC (0.2467 ± 0.003333 to 0.21 ± 0.03512) and SpDWC (0.24 ± 0 to 0.1267 ± 0.04177) decreased within the incubation period and were not significantly different from each other but significantly different from that of SpDW (0.36 ± 0.03) at p≤0.05. The optical densities of SpKW (0.2767 ± 0.006667 to 0.4667 ± 0.06119), SpC and SpKWC (0.24 ± 0 to 0.15 ± 0.05033) were all significantly different from each other. There was significant decrease (p≤0.05) in the optical densities of Salmonella cultures infected or/and treated with phage SpR (0.2467 ± 0.02028 to 0.2033 ± 0.08413), SpC and SpRC (0.24 ± 0.01155 to 0.68 ± 0.02082) compared to the control Sp but there was a change at the seventh hour where the optical density of SpRC started increasing and even superseded the control at the 17th hour and continued increasing steadily above the control. The OD600 of SpR was not significantly different from that of SpC but both were significantly different from that of SpRC at p≤0.05.

KEYWORDS: Salmonella Species, Antibiotics, Chloramphenicol, Phages

INTRODUCTION

Chemotherapeutic agents used to treat infectious disease destroy pathogenic microorganisms or inhibit their growth at concentrations low enough to avoid undesirable damage to the host [1,2]. Chloramphenicol a potent antibiotic with high efficacy was first produced from cultures of Streptomyces venezuelae but it is now synthesized chemically. Chloramphenicol is among the antibiotics whose mode of action is the inhibition of protein synthesis by binding to 23S rRNA on the 50S ribosomal subunit to inhibit the peptidyl transferase reaction. However, due to its high toxicity, it is used only in life-threatening situations when no other drug is adequate [1,3-5]. In addition to its high toxicity, there have been reports of dramatic rise in resistance to chloramphenicol by pathogenic bacteria such as Salmonella

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[6-9]. Efforts to overcome these challenges continue in scientific research and search for other approaches such as harnessing the great potential of Bacteriophages or simply, phages. Bacteriophages can be the best answer to antibiotic resistance in the treatment of bacterial infections [10-14]. These phages are considered to be economical, safe, self- replicating and are effective bactericidal agents [15,16]. A vital area of phage research that shows possibility of its deployment to control human pathogens such as Salmonella is its use to treat infections in animals as well as to prevent carriage of zoonotic pathogens that might subsequently get into the food chain system [17-20]. Clinical trials have been carried out with phage preparations to treat different infections in humans such as ear infection, burn wounds and leg ulcers which showed about 80% decline in mean bacterial cell count at infection site in phage-treated patients [21]. Other trials showed that phage can be used in combination with antibiotics to treat certain infections resistant to antibiotics alone [22]. Phage preparations have been administered to patients whose infections were unresponsive to antibiotics therapy. The results showed a high percentage full recovery (about 93%) and improvements in condition [23-25].

Though there are in-vivo trials that suggesting the use of antibiotics in combination with bacteriophages, to the best of our knowledge, this is the first published research in-vitro to assay the combined activity pattern of phages and an antibiotic (Chloramphenicol) on a bacterium (Salmonella Species) as well as compare and contrast their separate and combined activities. The aim of the study therefore is to determine the antibacterial activities of bacteriophages and chloramphenicol on Salmonella species as well as combined activities of bacteriophage and the antibiotics on the isolates.

MATERIALS AND METHODS

Bacterial and Phage Collection

Strains of Salmonella Salmonella species and Salmonella pullorum were obtained from the Department of Microbiology and confirmed using Commercial Identification kit. Similarly, two previously characterized bacteriophages (DW and KW) were also supplied for this research by the Department of Microbiology, Ahmadu Bello University, Zaria. A reference phage preparation 'Salmonelex' was obtained from Microes Food Safety (Netherlands) and used as reference phage stock

Assay for the Lytic Pattern of the Bacteriophages alone on the Salmonella

One mililitre of 10% suspension of 2×10^{11} pfu/ml of each phage (DW, KW and R) was added to 150ml of a 4 hour culture of the bacterial host cultivated in tryptone soya broth (TSB) in 200ml conical flasks. Half mililitre of CaCl2 solution was added and the flasks were incubated in a shaker rotating at 100 rpm for 24 hours at room temperature. Every hour, during the 24 hours incubation, the absorbance of the culture is measured at 600nm using a spectrophotometer. The negative control (sterile TSB) was used as the blank to calibrate the colorimeter before the each series of assay. The positive controls that contain only the bacterium and no phages were subjected to the same condition as the infected cultures [26-28].

Determination of the Activity of Chloramphenicol on the Salmonella species

One mililitre of Chloramphenicol (0.048g injectable dissolved in 10ml sterile distilled water) was added to each 150ml of the 4 hour old culture of the two isolates making it equivalent to 32μg/ml- and labelled C. All the mixtures and their positive controls were incubated at room temperature for 24 hours in a shaker rotating at 100 rpm during which the absorbance were measured hourly.

### Determination of the combined activity of phages and antibiotics on isolates in liquid medium

To each 150 ml of the 4 hour culture of either isolates in TSB, 1ml of 10% phage suspension, 0.5ml of CaCl2 solution and 1ml of chloramphenicol (32μg/ml) were added. They were labelled SsDWC (Combination of phage DW and Chloramphenicol), SpDWC (Combination of phage DW and Chloramphenicol), SsKWC (Combination of phage KW and Chloramphenicol), SpKWC (Combination of phage KW and Chloramphenicol), SsRC (Combination of phage R and Chloramphenicol), SpRC (Combination of phage R and Chloramphenicol), The mixtures were incubated at room temperature in a shaker rotating at 100 rpm for a 24 hour period during which the absorbance were measured hourly at OD of 600nm. The positive controls contain neither phage nor antibiotics.

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Data analysis

The activities of the bacteriophages and Chloramphenicol as well as the combination of the phages and Chloramphenicol where compared at p≤0.05 using one way Analysis of Variances (ANOVA) and Tukey Post Hoc test by International Business Machines SPSS Statistics version 21 and GraphPad Prism version 5.

RESULTS

The Optical Densities (OD) of both Salmonella species (Ss) and Salmonella Pollurum (Sp) growth after 24 hrs infection by phages remained low (SsDW-0.2 ± 0.0033 to 0.2 ± 0.11; SsKW-0.23 ± 0.015 to 0.37 ± 0.15 and SsR-0.25 ± 0.012 to 0.19 ± 0.11) while the uninfected controls maintain high (Ss-0.22 ± 0.015 to 0.64±0.055) (Figure 1). The decrease in OD600 of SsDW, SsR and SsKW were not significantly different from each other at p≤0.05. The difference in OD600 of SpDW (0.25 ±0.02309 to 0.36±0.03), SpKW (0.2767 ± 0.006667 to 0.4667± 0.06119) and SpR (SpR-0.2467 ± 0.02028 to 0.2033 ± 0.08413) were significantly different from each other at p≤0.05 except SpDW and 2SpKW which are not significantly different from each other but lower than the uninfected culture (Sp-0.2333 ± 0.02333 to 0.62 ± 0.02887). The peak for SpDW, SpKW and SpR were at the 2nd, 14th, 3rd and 1st hours respectively (Figure 2).
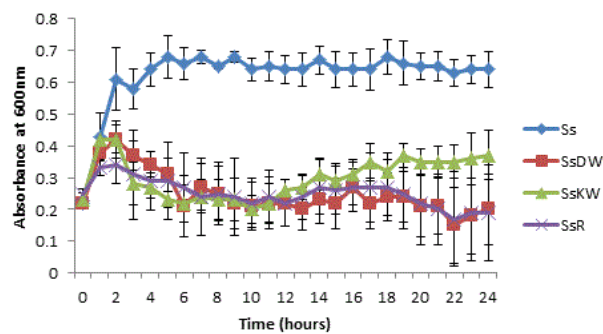
Figure 1: Optical Density (OD) development of uninfected control culture (Ss) and parallel cultures infected with phages SsDW, SsKW and SsR. The data represent the mean ± standard error
Keys: Ss-Control uninfected culture of Salmonella species, SsDW-Culture of Salmonella species infected with Phage DW, SsR-Culture of Salmonella species infected with Reference phage (Salmonelex), SsKW-Culture of Salmonella species infected with Phage KW.
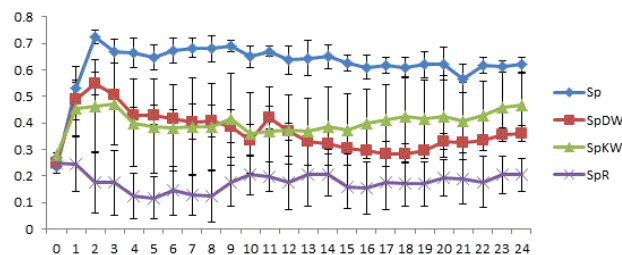
Figure 2: Optical Density (OD) development of uninfected control culture (Sp) and parallel cultures infected with phages 2DW, 2KS, 2KW and 2R. The data represent the mean ± standard error.
Keys: Sp-Control uninfected culture of Salmonella Pullorum, SpDW-Culture of Salmonella Pullorum infected with Phage DW, SpR-Culture of Salmonella Pullorum infected with Reference phage (Salmonelex), SpKW-Culture of Salmonella Pullorum infected with Phage KW

The absorbance of the SsDW (0.22 ± 0.0033 to 0.2 ± 0.11), SsC (0.23 ± 0.0033 to 0.17 ± 0.05) and the combination SsDWC (0.23 ± 0.01 to 0.37 ± 0.2) decreased significantly (p≤0.05) compared to that of Ss (0.22 ± 0.015 to 0.64 ± 0.055). The activity of DW and DWC were not significantly different from each other as shown in Figure 3. Figure 4 show that there were significant differences at p≤0.05 between the reductions due to Chloramphenicol (SsC-0.23 ± 0.0033 to 0.17 ± 0.05) and SsKW (0.23 ± 0.015 to 0.37 ± 0.15) compared to SsKWC (0.24 ± 0.01 to 0.14 ± 0.1). Between KW and C, the actions are not significantly different. There is no significant difference between the change in optical densities of SsR (0.25 ± 0.012 to 0.19 ± 0.11) and SsC (0.23 ± 0.0033 to 0.17 ± 0.05) and SsRC (0.23 ± 0.0033 to 0.17 ± 0.13) as shown in Figure 5.
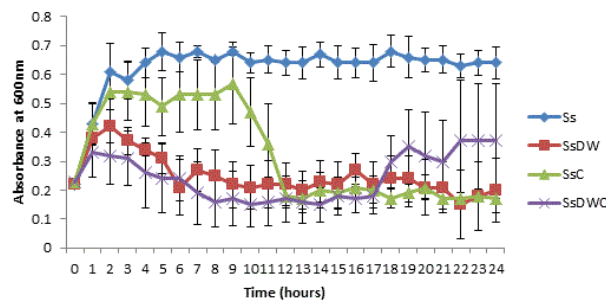
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Figure 3: Optical Density (OD) development of uninfected/untreated control culture (Ss) and parallel cultures infected/treated with DW, Chloramphenicol and both. The data represent the mean ± standard error.
Key: Ss-Control uninfected/untreated culture of Salmonella species, SsDW-Culture of Salmonella species infected with phage DW, SsC-Culture of Salmonella species treated with Chloramphenicol, SsDWC-Culture of Salmonella species infected and treated with both phage DW and Chloramphenicol
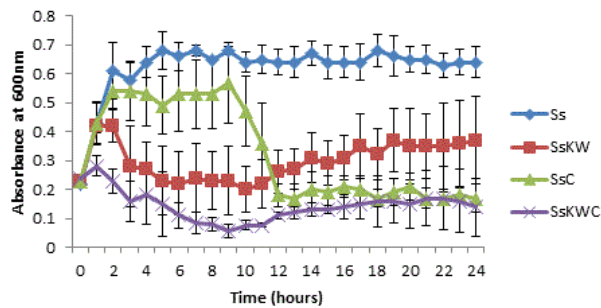

Figure 4: Optical Density (OD) development of uninfected control culture (Ss) and parallel cultures infected/treated with KW, Chloramphenicol and both. The data represent the mean ± standard error.
Key: Ss-Control uninfected/untreated culture of Salmonella species, SsKW-Culture of Salmonella species infected with phage KW, SsC-Culture of Salmonella species treated with Chloramphenicol, SsKWC-Culture of Salmonella species infected and treated with both phage KW and Chloramphenicol
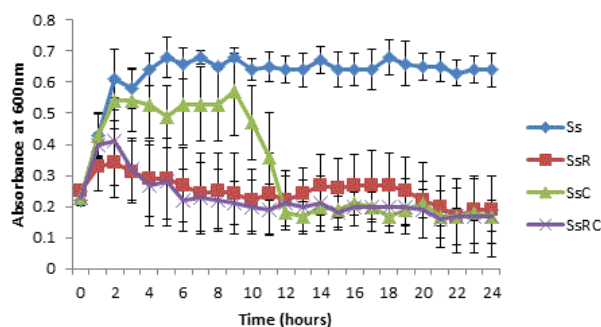

Figure 5: Optical Density (OD) development of uninfected control culture (Ss) and parallel cultures infected/treated with R, Chloramphenicol and both. The data represent the mean ± standard error.
Key: Ss-Control uninfected/untreated culture of Salmonella species, SsR-Culture of Salmonella species infected with phage R, SsC-Culture of Salmonella species treated with Chloramphenicol, SsRC-Culture of Salmonella species infected and treated with both phage R and Chloramphenicol

The optical densities of Sp increased significantly, being the control (0.2333 ± 0.02333 to 0.62 ± 0.02887) while SpC (0.2467 ± 0.003333 to 0.21 ± 0.03512) and SpDWC (0.24 ± 0 to 0.1267 ± 0.04177) decreased within the incubation period and were not significantly different from each other but both are significantly different from that of SpDW (0.25 ± 0.02309 to 0.36 ± 0.03) at $p \leq 0.05$ (Figure 6). The optical densities of SpKW (0.2767 ± 0.006667 to 0.4667 ± 0.06119), SpC and SpKWC (0.24 ± 0 to 0.15 ± 0.05033) were all significantly different from each other as shown in

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Figure 7. There was significant decrease (p≤0.05) in the optical densities of Salmonella cultures infected or/and treated with phage SpR (0.2467 ± 0.02028 to 0.2033 ± 0.08413), SpC and SpRC (0.24 ± 0.01155 to 0.68 ± 0.02082) compared to the control Sp but there was a change at the seventh hour where the optical density of SpRC started increasing and even superseded the control at the 17th hour and continued increasing steadily above the control as shown in Figure 8. The OD600 of SpR was not significantly different from that of SpC but both were significantly different from that of SpRC at p≤0.05.

Figure 6: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2DW, Chloramphenicol and both. The data represent the mean ± standard error.
Key: S2-Control uninfected/untreated culture of Salmonella Pullorum, 2DW-Culture of Salmonella Pullorum infected with phage 2DW, 2C-Culture of Salmonella Pullorum treated with Chloramphenicol, 2DWC-Culture of Salmonella Pullorum infected and treated with both phage 2DW and Chloramphenicol
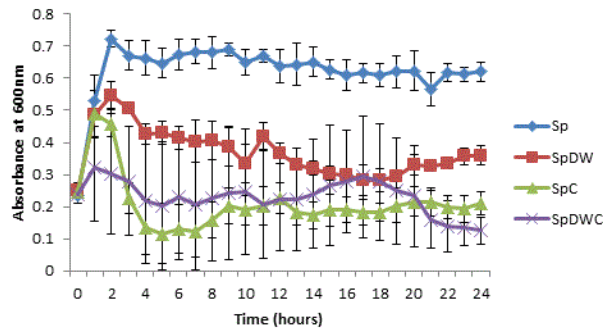

Figure 7: Optical Density (OD) development of uninfected control culture (Sp) and parallel cultures infected/treated with KW, Chloramphenicol and both. The data represent the mean ± standard error.
Key: Sp-Control uninfected/untreated culture of Salmonella Pullorum, SpKW-Culture of Salmonella Pullorum infected with phage KW, SpC-Culture of Salmonella Pullorum treated with Chloramphenicol, SpKWC-Culture of Salmonella Pullorum infected and treated with both phage KW and Chloramphenicol
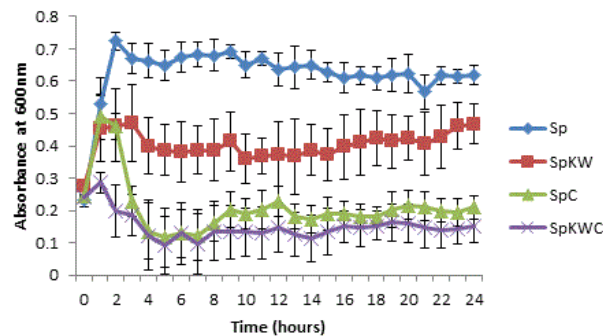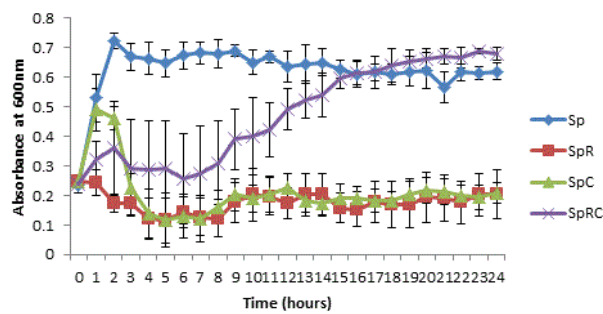
Figure 8: Optical Density (OD) development of uninfected control culture (Sp) and parallel cultures infected/treated with R, Chloramphenicol and both. The data represent the mean ± standard error.

Key: Sp-Control uninfected/untreated culture of Salmonella Pullorum, SpR-Culture of Salmonella Pullorum infected with phage R, SpC-Culture of Salmonella Pullorum treated with Chloramphenicol, SpRC-Culture of Salmonella Pullorum infected and treated with both phage R and Chloramphenicol

DISCUSSION

The study showed the effectiveness of the phages in vitro as biological antibacterial agents against Salmonella species (Ss) and Salmonella Pullorum (Sp). The antibacterial patterns of the phages in this study are similar to findings of Chibani-Chennoufi et al. (2004) [26], Atterbury et al. (2007) [27], Synnott et al. (2009) [28], Hungaro et al. (2013)[29] and Piracha et al. (2014) [30] who all observed similar trends (significant decrease in OD) on several phage treated bacterial isolates compared to their respective untreated controls. The significant reduction in OD600 of cultures of the isolates treated with Chloramphenicol compared to the controls also indicates the susceptibilities of the isolates to the antibiotic even in liquid medium. Considering the significant reduction in OD600 of the infected/ treated cultures in comparison to the uninfected/untreated cultures as the lytic activity of the phages on the host and likewise the detrimental effects of the antibiotics on the host, deductions were made that all the bacteriophages, the antibiotic and the combinations of the phages and antibiotic-except SpRC, had antibacterial effects on the two salmonellae used in this study.

The phage DW, Chloramphenicol and their combination DWC all had good observable effects on Ss. Also, they have similar degree of activity. In the case of KW, the combination of the phage and chloramphenicol (KWC) has better effect compared to the phage alone or antibiotic alone. For 1R in relation to Chloramphenicol, the phage alone served as the best option compared to the antibiotic alone or the combination of the phage and the antibiotic. In the case of Sp, DW, the antibiotic and their combination can serve as control agents for Sp except that in this case, Chloramphenicol and phage combination DWC is better options in performance than the phage DW alone. As expected and unlike the previous results so far -except for the case of R, chloramphenicol and their combination, the combination of KW with the antibiotic produce synergistic effect which made it better potential control agent than the phage alone or antibiotic alone. In the case of the reference phage on Sp R, there was a peculiarity observed where OD600 of culture infected and treated with combination of phage and chloramphenicol started increasing at a point till it exceeded the control. The reason for this pattern is unknown since it didn't happen in the case of the phage alone or the antibiotic alone but it could be attributed to resistance to both phage and antibiotic which could result from mutation or adaptation of the host bacterium as replication continues due to processes such as transduction, development of resistant genes and loss of receptors and colonization factors for both phage and antibiotic [30,31]. This can be a hindrance in using such approach for combination therapy and control measures. This resistance exhibited calls for caution in the use of phages in combination with antibiotics for control of Salmonella in different fields of application. The other results for R alone and Chloramphenicol alone show that either of them can be used since there is no significant difference between them.

CONCLUSION

The activity pattern of the phages on their respective Salmonella host indicates the ability of virulent phages to lyse Salmonella species and therefore their use in the control of Salmonella Species. They were seen as potential biocontrol agents that can be used against pathogens such as Salmonella for preventive, therapeutic and curative measures in the fields of food, industries, biosanitation, agriculture, veterinary and human medicine. Base on the results and deductions from this research, phages were seen to perform well like antibiotics -and in some cases more,

in the control of the Salmonella species. They were also seen to be efficient either alone or in combination with antibiotics. They can therefore be used as alternative to antibiotics or in combination with antibiotics in cases of adverse side effects to antibiotics, resistance to antibiotics, financial constrains to access antibiotics.

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