Section A-Research paper

Combating Antimicrobial Resistance: The Role of Bacteriophage in Controlling Sewage Associated Salmonella Pathogens in Tirunelveli District, Tamil Nadu, India

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Abstract

The increase in the availability of new antibiotics has led to the eradication of certain pathogens, but it has also resulted in the emergence of diseases caused by antimicrobial resistant bacteria. The World Health Organization (WHO) estimates that approximately 700,000 people die annually worldwide due to antimicrobial resistance (AMR). If AMR is not effectively addressed, the mortality rate could rise to 100 million per year by 2050. This study aims to eliminate the source of Salmonella bacteria, which causes Typhoid, in sewage and assess the society's awareness of AMR. An online survey was conducted using a Google form to gather information. Sewage samples were collected from six different locations and Salmonella bacteria were isolated. Bacteriophage, a virus that infects bacteria, was used to eradicate the isolated Salmonella bacteria. The enumeration of Salmonella bacteria in selected samples was carried out using the Most Probable Number (MPN) technique, approved by the International Organization for Standardization (ISO), and the isolated species were confirmed through biochemical tests. The results showed that the MPN per 100 ml of Salmonella bacteria was 1100 before the phage treatment, but after the treatment, it decreased to 3. These findings suggest that bacteriophages can serve as an alternative method for controlling infectious diseases.

Keywords

Bacteriophages; Salmonella; antibiotics; infectious diseases.

Introduction

The emergence of antibiotics brought about a significant relief from the health problems associated with microorganisms (1). For many years, antibiotics proved to be highly effective in combating various bacterial infections and saving countless lives (2). They revolutionized medical treatment, providing a powerful tool to combat infectious diseases (3).

However, this era of antibiotic efficacy came to an end as the medical community discovered a concerning trend. Microorganisms, specifically bacteria, began to develop resistance to antibiotics (4). It was during the late 1950s and early 1960s that antibiotic resistance to multiple antimicrobial agents was detected for the first time among enteric bacteria, including Salmonella, Shigella, and Escherichia coli (5). These resistant strains caused significant clinical, economic losses, and loss of life, particularly in the developing world.

The rise of antibiotic resistance can be attributed to several factors. One primary factor is the increasing use of antimicrobials, both in healthcare settings and in the community (6). Antibiotics were readily available without a prescription, particularly in developing countries, leading to their indiscriminate use. The misuse and overuse of antibiotics accelerated the incidence of resistance (7). Moreover, inadequate hygiene settings and limited healthcare resources facilitated the transmission of resistance, creating a breeding ground for resistant bacteria to thrive (8).

The gravity of the situation became apparent when the World Health Organization (WHO) highlighted the threat posed by antimicrobial resistance. Global deaths attributed to bacterial AMR by pathogen-drug combinations surpassed mortality rates associated with certain diseases. It became evident that AMR is a ticking time bomb, with the potential to cause devastating consequences in the future (9).

In response to the crisis, the WHO spearheaded efforts to address antimicrobial resistance. The organization developed and implemented several global action plans on AMR, emphasizing the importance of antibiotic stewardship programs in healthcare facilities, particularly in low and middle-income countries (10, 11). These programs aimed to promote responsible and appropriate antibiotic usage and disseminate educational resources and toolkits for AMR management in healthcare settings.

Salmonella, the bacteria responsible for causing typhoid, has been recognized as a top priority pathogen by the World Health Organization since 2017. It poses a significant threat to public health worldwide. Consequently, this study aims to focus on the identification, isolation, and treatment of the typhoid-causing bacteria using bacteriophage therapy (12, 13).

Bacteriophages are viruses that specifically infect and kill bacteria. By targeting and eradicating the specific strains of Salmonella prevalent in the environment, bacteriophage therapy offers a promising alternative approach to reduce antimicrobial resistance (14). The study aims to evaluate the effectiveness of bacteriophage treatment in eliminating Salmonella bacteria and reducing their prevalence in the sewage samples collected from different locations.

To quantify the impact of bacteriophage therapy, the Most Probable Number (MPN) technique, approved by the International Organization for Standardization (ISO) (15), is used to enumerate the Salmonella bacteria in selected samples. This technique provides an estimation of bacterial counts per 100 ml of the sewage sample. Prior to the phage treatment, the MPN for Salmonella is observed to be 1100, indicating a high bacterial load. However, after the treatment, the MPN dramatically decreases to 3, demonstrating the potential of bacteriophage therapy in controlling infectious diseases caused by Salmonella.

The study also highlights the critical need for increased awareness and understanding of antimicrobial resistance among society. An online survey was conducted using a Google form to assess the awareness of AMR among individuals. Understanding the level of knowledge and perception about AMR is crucial for designing effective interventions and educational campaigns to promote responsible antibiotic use.

Despite the alarming statistics and the urgent need for action, there are significant challenges in addressing antimicrobial resistance. Inadequate surveillance systems and limited laboratory antimicrobial susceptibility testing capabilities hinder the ability to track resistance trends accurately and develop evidence-based policies. Particularly in rural areas, the lack of capacity in performing routine antimicrobial susceptibility testing poses a major obstacle.

In the absence of patient-specific antimicrobial susceptibility testing, community-based antimicrobial surveillance data can provide valuable insights to healthcare professionals in specific regions or communities. Regular and continuous surveillance is necessary since resistance rates can vary both geographically and over time within a country. By monitoring resistance patterns, healthcare providers can make informed decisions about appropriate antimicrobial treatment options.

Hence the present study mainly focuses on the complete eradication of the source by combating nature of the bacteriophage of the specific resisted bacteria. This study also includes the survey on the awareness of AMR.

Materials and methods

Principle

Enumerate the number of Salmonella species using MPN (Most Probable Number) technique. The bacteria were enriched by Rappaport Vassiliadis Soya Broth (RV) which is a selective enrichment medium for the salmonella species. Then the bacteria were cultured in the Xylose Lysine Deoxycholate agar a selective growth medium used for the isolation of the Salmonella species and Shigella species from the samples. Simultaneously bacteriophage is added to the collected sample and the analysis for the salmonella species was carried out. Finally, the biochemical tests were carried out for the confirmation of the selective cultured bacteria on the basis of their biochemical activities.

Materials

1liter Sterile container for sample collection, Sterile R.V. Broth Tubes,Sterile Surface dried XLD Agar, Salmonella Phage, Petri Plates,Sterile Pipettes (Glass), Sterile Saline,Sterile Nutrient Agar Slant and Biochemical Identification kits HimediaCatlog No. KB011.

Procedure

Sample Collection

Aseptically the samples were collected from the Sewage at different locations (Figure 1) as planned and transported to the lab in cool box.

Sample 1: It was collected near Tirunelveli police canteen which is located at PP7R+HFJ, Palayamkottai, Tirunelveli, Tamil Nadu 627007, Latitude 8.714096^{o} Longitude 77.741117^{o}.

Sample 2: It was collected near new bus stand which is located at Trivandrum Rd, near new bus stand, Vasanthanagar, Tirunelveli Tamil Nadu; Latitude 8.700366^{o} Longitude 77.728626^{o}

Sample 3: It was collected from perumalpuram located at L223, Noble ST, Vasanth Nagar, Tirunelveli, Tamil Nadu 627007, India Latitude 8.707629^{o} Longitude 77.743176^{o}

Sample 4: It was collected from MQR3+RX, Thirumal Nagar, Tirunelveli, Tamil Nadu 627007, Latitude 8.69214^{o} Longitude 77.7554^{o}.

Sample 5: It was collected Near Chennai Silks located at 63, Trivandrum Rd, Palayamkottai, Tirunelveli, Tamil Nadu Latitude 77.721177^{o} Longitude 8.727717^{o}.

Sample 6: It is collected Near ESIC hospital located PPHC+Q3Q, Vanarpettai, Tirunelveli, Tamil Nadu 627003, Latitude 77.720047^{o} Longitude 8.729329^{o}.

Inoculation

The RV broth were filled in each test tube as double strength of 10ml, single strength of 10ml and single strength of 9ml for dilution. Three test tubes of D.S (double strength), seven test tubes of S.S (single strength) and two test tubes of S.S for dilution were set aside. To this D.S and S.S test tubes with RV broth (Three test tubes each) 1ml of sample was inoculated aseptically and 1ml to the S.S tube of 9ml from which 1ml of the dilution is mixed in RV broth. These test tubes were set for incubation for 24 hours at 35^{o}C. Meanwhile for the phage therapy 4ml of concentrated phage is added to the sterile water of 1000ml. Now from this 10ml of diluted *Salmonella* phage is added to the sample at room temperature for two hours. Aseptically the samples were inoculated in RV broth as mentioned earlier and were set for incubation.

Plating and Streaking

After the incubation the positive results to be noted are set aside. For plating, the sterile XLD agar is poured in the sterile Petri plate aseptically and is kept for 15 minutes. Once the agar solidifies the plates are kept for surface drying for an hour and are now ready for streaking. The sterile XLD plates are streaked with two loops full of culture aseptically. These plates are incubated at 35 ^{o}C for 24 hours. If the colonies are not found, they are to be incubated again for 24 hours.

Biochemical tests

The plates were examined for the suspected colonies and the biochemical tests for the suspected colonies to be analysed was sent to West Coast Frozen food laboratory along with the sample mapping. Biochemical Identification was done by "KB011 HiSalmonella" Identification Kit. It is a standardized colorimetric identification method which utilized the carbohydrates within the wells for biochemical analysis. The tests utilize the principle of pH change and substrate utilization to detect the presence of Salmonella. During incubation, Salmonella

undergoes metabolic changes that result in a noticeable color change in the media. This color change can be interpreted visually or by adding a specific reagent to aid in the detection process. Inoculate each well with 50 µl of the culture 4 – 6 hrs and incubate at 37^{o}C for 18 – 24 hours. After incubation add the given reagent in well No.1 and No.2 (standard) before reading the results. Wells are visually observed. All results to be recorded and final results to be confirmed with comparative chart given by Himedia with KB011 kit.

Survey

In order to assess the awareness of AMR among the society, survey was conducted through online via Google form. The survey mainly considered the answers given by the respective sectors to know the awareness among the population surveyed. The sectors were grouped into different categories which includes Students, Graduates, Teachers/Assistant professor, Medical and Food science and other professions.

Result and discussion

The untreated samples within the RV broth after incubation turns from blue colour to colourless this indicate the presence of the *salmonella* bacteria (Figure 2). From each samples the colourless tubes were noted for each dilution. According to the morphology of the bacteria (red colour with Black centre) the numbers of plates were noted. All the samples collected from the selected study area indicated the presence of *Salmonella* bacteria (Figure 3).

The treated samples (Introducing phage into the sample) within the RV broth after incubation remains the same (Blue colour) this indicates the absence of the salmonella bacteria as the Salmonella bacteriophage minimizes the antimicrobial resistant gene of the bacteria from transmitting (Figure 2). Among the six samples only in sample 2 of 0.1ml S.S (single strength) two test tubes was colourless. To check the presumptive positive three plates for each sample were streaked. In the XLD streaked plate only the sample 2 of 0.1ml S.S had colonies.

The biochemical results given by West Coast Frozen foods laboratory for the suspected colonies were consolidated. The enumeration of the isolated colonies was noted by taking two tables in consideration and the MPN table. For each samples the number of positive tubes and the positive plates are matched with the MPN table for the count. The Enumeration was noted for the both the treated and the untreated samples (Table 1 and Table 2).

Figure 5 depicts the difference within the untreated and treated sample. Before phage treatment the MPN range for *Salmonella* was found to be from 21 to 1100 but once the samples were treated with the *Salmonella* bacteriophage it was found that the peak of AMR bacteria tend to reduce. Only one sample which indicated the presence of *Salmonella* bacteria after treatment was analyzed (sample 2 of 0.1ml S.S dilution).

Therefore it was found that Bacteriophage treatment can aid in reducing the *Salmonella* bacteria (Figure 4). Phage therapy should be considered and put forth into action. This implies that bacteriophage treatment can efficiently combat against the emerging AMR. Apart from the protocols and the strategies bacteriophage therapy can rapidly control the AMR.

From the survey (Figure 11) conducted among the different sectors, seventy responses were received from the survey. The responses were segregated according to the respective sectors (Figure 6 to Figure 10)

A very low knowledge level was recorded among the school students about 96% were unaware (Figure 6) and among other professions 83 % were unaware (Figure 9), comparatively peoples in medical and food science had a well-developed knowledge regarding AMR nearly 82 % had awareness on AMR (Figure 8).

In case of Graduates 67 % were unaware (Figure 7) among and teachers' sector 65 % were unaware (Figure 10), most of them were able to answer the basic questions about AMR but were unaware of its impact on human health. This shows that still awareness on AMR is mandatory. Programmes and policies on AMR should be established for the targeted sector. The sectors with low knowledge level about AMR are the target for the awareness programme.

Conclusion

Antimicrobial resistance has emerged as a global threat to the human health; action must be taken in to consideration for combating against AMR. As the bacteriophage has the combating nature against the AMR, bacteriophage therapy must be taken into consideration. This treatment can significantly minimize the emergence of the antimicrobial resistant bacteria. Along with the treatment, the awareness on AMR must be carried out so that people will have the knowledge about the critical nature of AMR. New economic incentives need to be established for implementing the bacteriophage therapy which will be an effective tool to combat the antimicrobial resisted bacteria across countries.

The consequences of ineffective antibiotic management and the rise of antimicrobial resistance are already evident. Lives are being lost, and patients are suffering due to the lack of effective treatment options. Immediate action is crucial to address this global health crisis. Governments, policymakers, healthcare providers, and the pharmaceutical industry must collaborate to develop and implement strategies that promote responsible antibiotic use, strengthen surveillance systems, and support the development of novel and effective antimicrobial therapies.

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Table(s)

Sl. No.	Sample No.	Source of the Sample	MPN Result (+ number of Tubes)			Positive Result in XLD			MPN Final Result (+ number of Tubes)			MPN / 100 ml from MPN Table
			10 ml DS	1 ml SS	0.1 ml SS	10 ml DS	1 ml SS	0.1 ml SS	10 ml DS	1 ml SS	0.1 ml SS	
1	S - 01	Tirunelveli police canteen	2	3	2	2	3	2	2	2	0	21
2	S - 02	Near New Bus stand	3	3	3	3	3	3	3	3	2	1100
3	S - 03	Perumalpuram	3	3	2	3	3	2	3	3	1	460

4	S - 04	Thirumal Nagar	3	3	3	3	2	3	3	2	2	210
5	S - 05	Near Chennai Silks	2	3	2	2	2	1	2	2	1	28
6	S - 06	Near ESIC Hospital	3	3	3	3	3	3	3	3	2	1100

Table - 1 Samples untreated with Salmonella Phage.

Sl. No.	**Sample No.**	**Source of the Sample**	**MPN Result (+ number of Tubes)**			**Positive Result in XLD**			**MPN Final Result (+ number of Tubes)**			**MPN / 100 ml from MPN Table**
			10 ml DS	**1 ml SS**	**0.1 ml SS**	**10 ml DS**	**1 ml SS**	**0.1 ml SS**	**10 ml DS**	**1 ml SS**	**0.1 ml SS**	
1	T - 01	Tirunelveli police canteen	0	0	0	0	0	0	0	0	0	0
2	T - 02	Near New Bus stand	0	0	2	0	0	2	0	0	1	3
3	T - 03	Perumalpuram	0	0	0	0	0	0	0	0	0	0
4	T - 04	Thirumal Nagar	0	0	0	0	0	0	0	0	0	0
5	T - 05	Near Chennai Silks	0	0	0	0	0	0	0	0	0	0
6	T - 06	Near ESIC Hospital	0	0	0	0	0	0	0	0	0	0

Table - 2: Samples after *Salmonella* Phage treatment.

Figure(s)

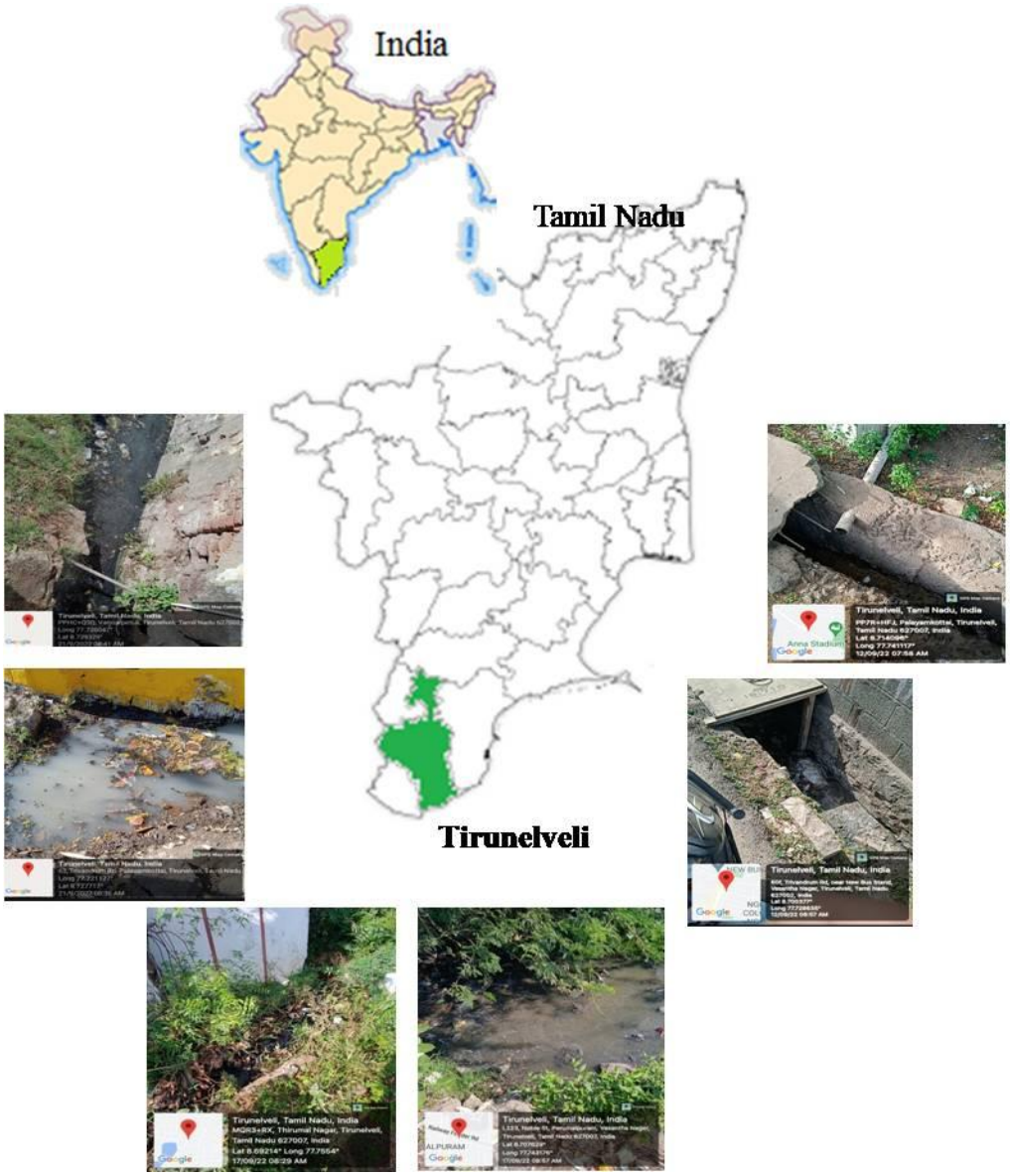

Figure 1: Sampling locations.

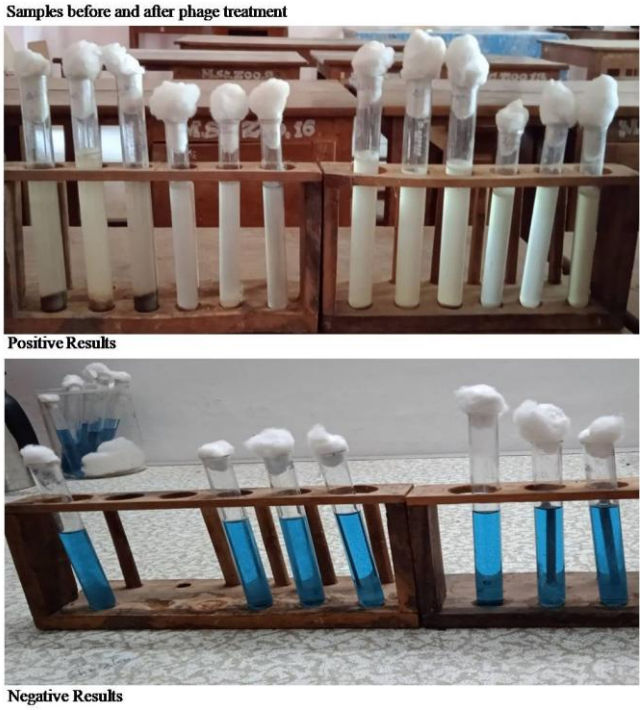

Figure 2: Positive and Negative results of phage treated samples.

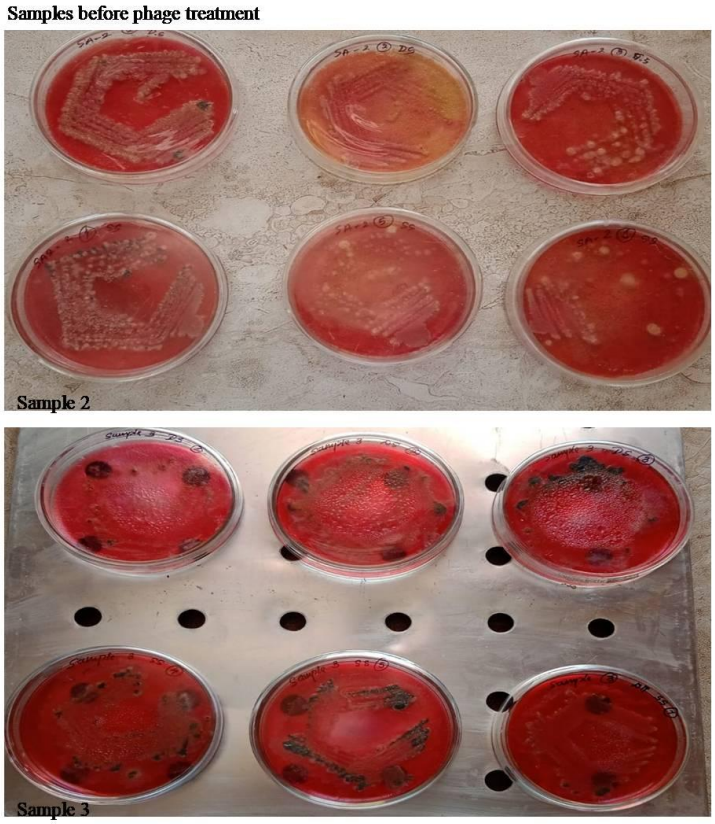

Figure 3: *Salmonella* culture plates on specific media in before phage treatment.

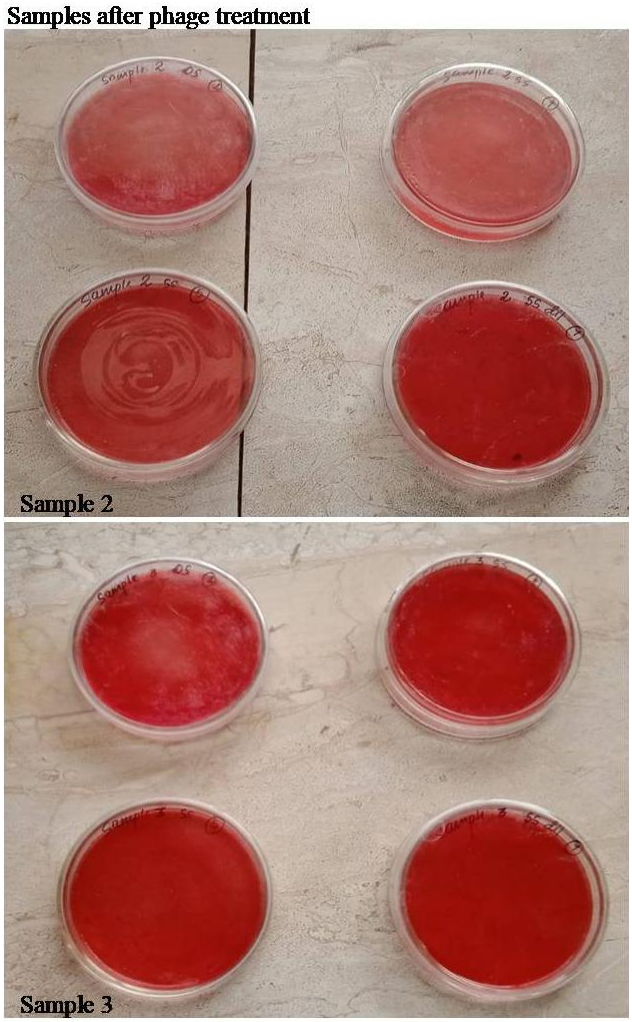

Figure 4: *Salmonella* culture plates on specific media in after phage treatment.

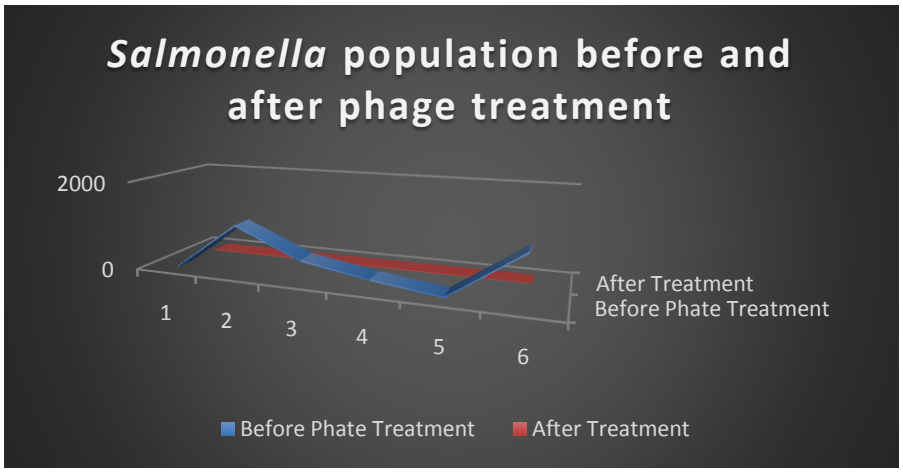

Figure 5: Variation in *Salmonella* population before and after phage treatment.

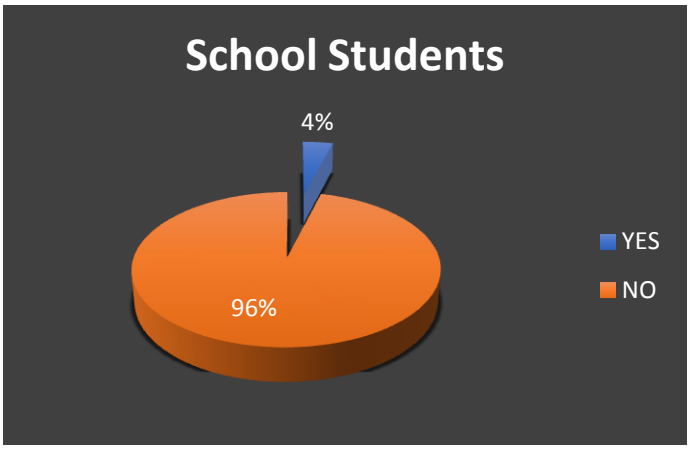

Figure 6: Pie diagram representing the percentage of awareness among School Students.

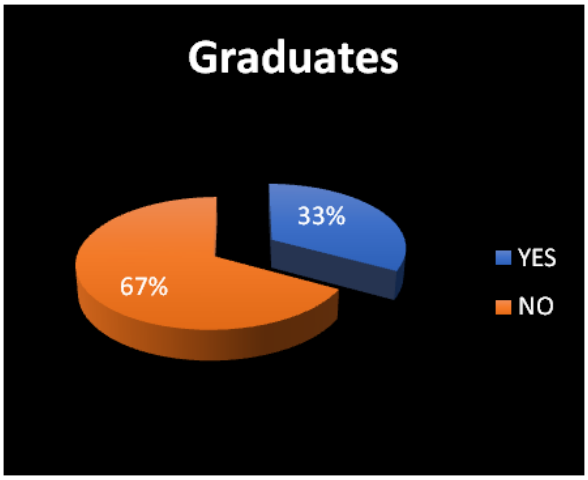

Figure 7: Pie diagram representing the percentage of awareness among graduates.

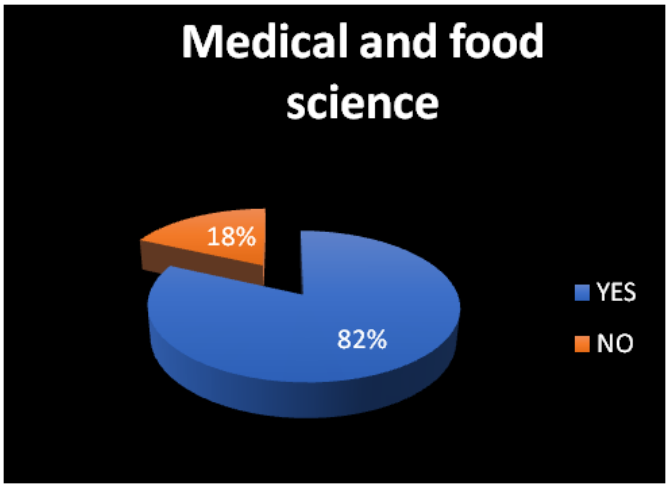

Figure 8: Pie diagram representing the percentage of awareness among Medical & Food Science.

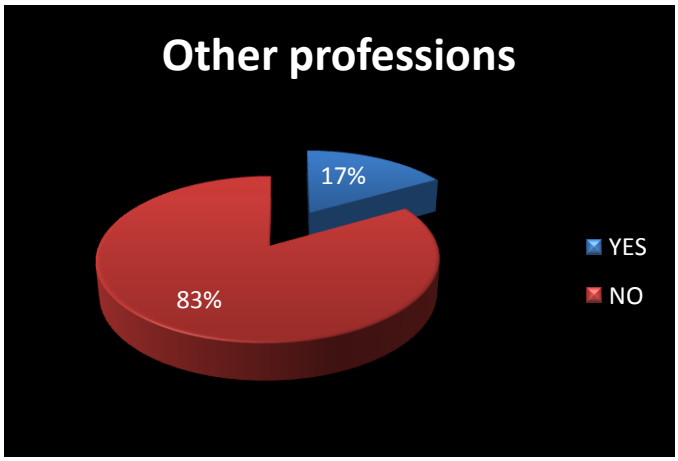

Figure 9: Pie diagram representing the AMR among Other Professions in %.

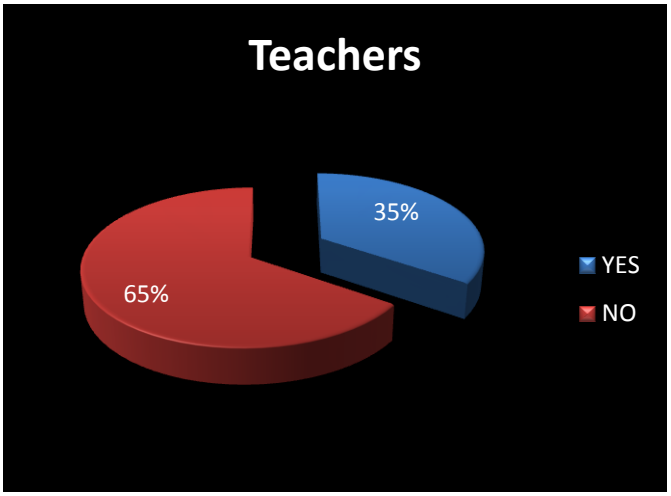

Figure 10: Pie diagram representing the awareness of awareness of AMR among Teachers in %.

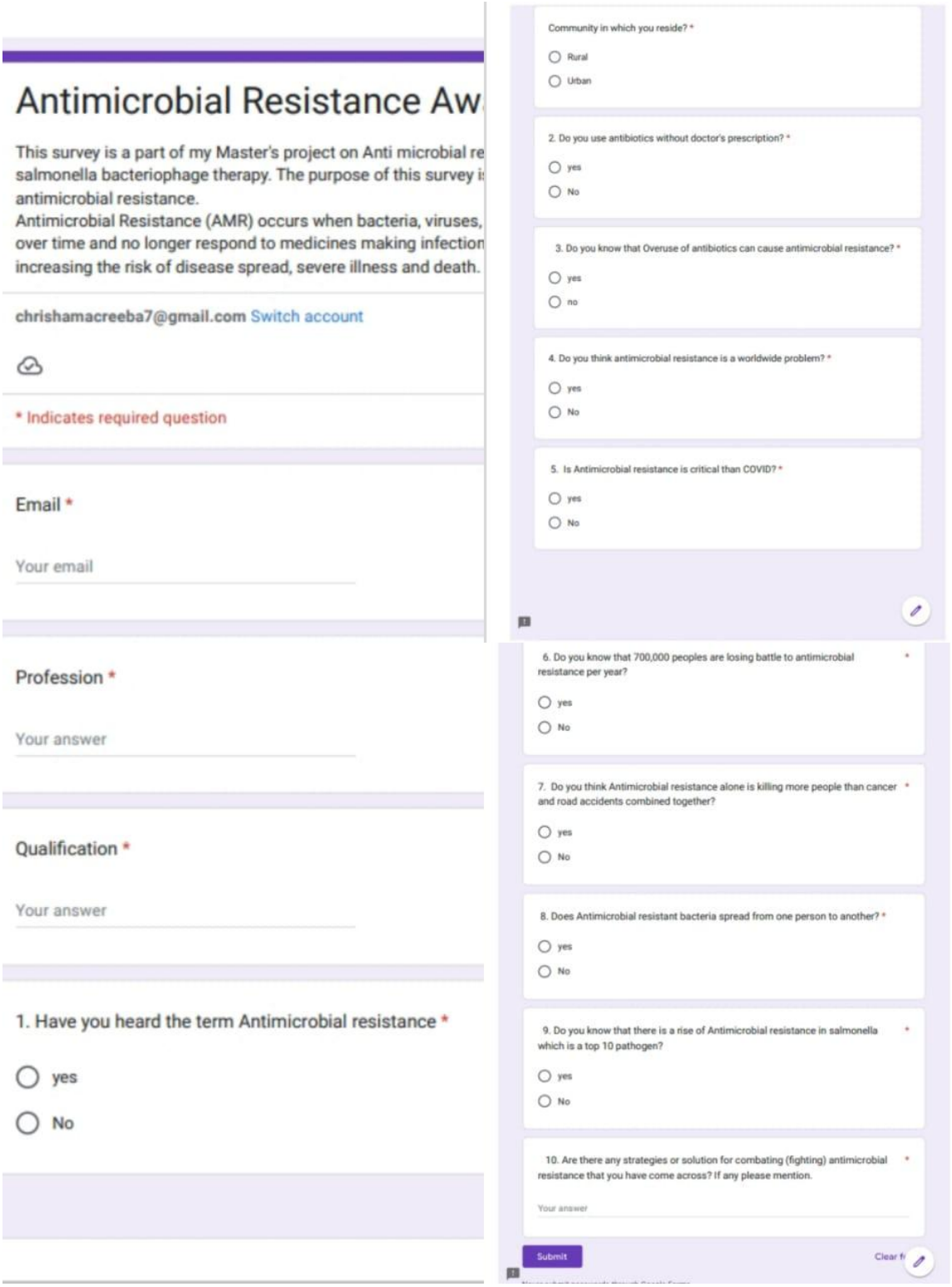

Figure 11: Google Survey form.