Introduction

Poultry infections like Salmonellosis, Colibacillosis are more common in Bangladesh. Antibiotics are randomly used for treatment, control & prevention of disease in poultry & also as a growth promotor which ultimately has an adverse effects on human health (Noor et al., 2020). As a result, nowadays antibiotic resistance & multidrug resistance has become very big issue which needs to be controlled (Aslam et al., 2018). To overcome this situation alternatives to antibiotics need to be searched (Joerger, 2003). One of the emerging drugs that can be used alternatives to antibiotics is bacteriophage therapy (Noor et al., 2020).

Bacteriophages are known as a group of obligate intracellular parasites that infects bacteria in a specific manner & can be easily manipulated. They can be found in all environment as long as their host are existed. It genome is ss/ds DNA or RNA. The majority of the identified phages are classified under the order caudovirales (ds DNA with tailed morphology) which includes three families Siphoviridae, Myoviridae, & Podoviridae (Fokine and Rossmann., 2014). Replication of phage viruses is similar to other viruses. After attachment to a specific receptor in the bacterial cell wall, the phage genome enters the cell. The capsid protein are usually stripped off & remains outside the cell. After entry into the cell many phage genomes are degraded & destroyed. Since phage are so prevalent in the environment, bacteria have specific mechanism to protect themselves against infection with phage restrictions / modification systems which depends on the recognition & destruction of foreign DNA. “Virulent” phages do not integrate their genetic material into host cell chromosome & kill the host cells (lytic infections). So these viruses may be used for destruction of bacteria for treatment purposes. Whereas “temperate ”phages integrate into the host DNA causing “Lysogeny” (Qadir, 2015).

Phage have many positive attributes as antimicrobials. Phage show more host specificity, & in most case show specificity to a single species or strain of bacteria, whereas antibiotics show broad spectrum activity, affected more than the targeted

organism. Phage don’t cause side effects but antibiotics cause many side effects including allergies & intestinal disorder.

Again antibiotics resistance occurs & is not limited to targeted bacteria whereas phage resistance occurs but it can be linked to host virulence attenuation. Also, phage can co-evolve with host. Development process of an antibiotics is time consuming than phage therapy development **(** Semler et al., 2012).

Objective: In Bangladesh phage solution are imported from foreign countries which cost a lot. Again due to its high cost most of the farmer show unwillingness to buy & use it. So, the objectives of the study are following :

1. To develop a phage preparation from locally available low cost source.
2. To develop a phage preparation to combat poultry infections.

Literature review

The use of bacteriophages to eliminate pathogen, mostly foodborne pathogens like *Salmonella*, *Camphylobacto*r & *E coli* seems quite promising as bacteriophages are present in every ecosystem & their number is 10 times more than the number of characterized bacteria.

According to Noor et al. (2020) dietary supplementation of bacteriophages at 0.5g/ kg feed significantly increased feed efficiency in broilers & also increased egg production in laying hens. It also decreased *Ecoli* &*Salmonella* colonization in intestine.

According to Tawakol et al. (2019) showed that bacteriophage treatment (by intra-tracheal inoculation) reduced the severity & prevented mortality of not only single APEC infection but also a mixed infection with APEC & Infectious Bronchitis virus.

According to another study (Wernicki et al., 2017) at a time of growing antibiotic resistance in bacteria & the resulting restrictions on the use of antibiotics, bacteriophages can provide an alternative means of eliminating pathogen.

According to Zhao et al. (2012) the inclusion of bacteriophages in poultry ration could benefit the poultry farmers in terms of improved feed conversion ratio in broilers & increased egg quality in layer.

In another study of Lim et al. (2011) it said that a positive effect of bacteriophages also observed in reducing mortality of chickens.

Huff et al. (2002) have demonstrated that aerosol spray of bacteriophages administered to 7 day old chicken prior to the triple challenge with *E coli* prevent airsaculitis caused by *Ecoli*.

According to Toro et al. (2005) the inclusion of bacteriophages could successfully reduce the *Salmonella* & *Ecoli* counts in chicken internal organs & feces.

Methods & Materials

For producing phage probiotic we had to first calculate phage concentration in per ml of phage culture. We have taken a T2 phage culture& grow phage by using following steps :

Step 1: Collection of blood from sheep.

Step 2: Blood agar preparation.

Step 3: *E coli* streaking in blood agar.

Step 4: Brain heart infusion broth preparation.

Step 5: *Ecoli* culture in brain heart infusion broth.

Step 6: Trypton broth preparation.

Step 7: Serial dilution of phage culture.

Step 8: Muller Hinton agar preparation.

Step 9: Inoculation of *E coli*& diluted phage culture in Muller Hinton agar.

Phage cannot grow without host cell. As the phage culture was a culture of T2 phage virus. We took *E coli* as a host to grow phage. I have taken permission from the head of Microbiology & Public health Department honorable Professor Dr. Abdul Ahad sir for doing lab work in microbiology lab. I have discussed my topic with my supervisor & also collected information from internet. At first I have made a protocol to do plaque assay. I have shown this protocol to our honorable teachers & by their consent I have started my lab work.

Step 1: Blood collection from sheep.

Equipments:

1. 70% alcohol
2. Scissors
3. Cotton
4. Two syringe of 12 ml with EDTA
5. 18 gauge needle

I have taken permission from Director of farm respected Associate Professor Dr. Md. Saiful Bari sir to collect blood.

Procedure:

1. At first the animal was restrained with head elevated & jugular vein exposed.
2. A small area over the jugular groove were clipped & swiped with antiseptic gauge to remove superficial dirt & debris. It also assists in visualizing raised vein.
3. Then I have occluded the jugular vein by applying pressure at the base of the jugular groove & visualize raised vein.
4. With bevel up, inserted needle through skin & into vein at 20 degreeangle & blood collected.
5. After complete collection, pressure was released to the vein applied pressure over injection site with gauge & the needle was removed.
6. The syringe was kept in refrigerator at 4 degree Celsius until use for further work.


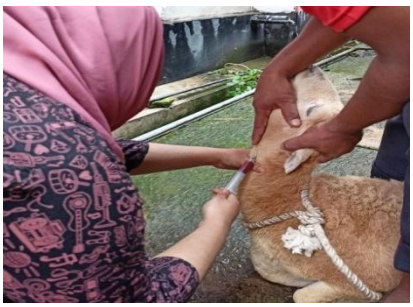

Figure 1: Blood collection from sheep

Step 2:Blood agar preparation.

Composition of Blood Agar:

- 0.5% Peptone
- 0.3% beef extract/yeast extract
- 1.5% agar
- 0.5% NaCl
- Distilled water
- (Since Blood Agar is made from Nutrient Agar, above is the composition of Nutrient Agar)
- 5% Sheep Blood
- pH should be from 7.2 to 7.6 (7.4)

Equipments:

1. Blood agar powder
2. Aluminium foil paper
3. Spatula
4. Distilled water
5. Conical flask
6. Heater
7. Agar plate
8. Sheep blood

Procedure:

1. 8 gram of nutrient agar powder was suspended in 200 ml of distilled water.
2. The mixture was then to heated to dissolve all components properly.
3. Then the mixture was autoclaved at 121 degree Celsius for 15 minutes.
4. Once the nutrient agar has been autoclaved, it was allowed to cool but not solidify.
5. When the agar was cooled to 45-50 degree Celsius, 5% (v/v) sterile defebrinated blood that has been warmed to room temperature was added & mixed gently but well.
6. Air bubbles were avoided.
7. Then mixture was dispensed into sterile agar plates while liquid.
8. Then the plates kept in the incubator at 37degree Celsius for 24 hours.

Figure 2: Blood agar preparation

Step 3: *E coli* streaking in blood agar

Equipments:

1. *Ecoli* Sample
2. Inoculating loop
3. 70% alcohol
4. Bunsen burner
5. Biosafety cabinet

Procedure:

1. Next day the blood agar were checked to find out if there is any contamination have or not.
2. Two *Ecoli* sample were given by respected sir assistant professor Dr. Eaftekhar Ahmed Rana Sir.
3. Before giving streaking biosafety cabinet area were disinfected through using 70% alcohol. Hands were also sterilized with alcohol. Inoculating loop was sterilized by placing it at an angle over a flame.
4. The vial containing sample was uncapped.
5. Then the loop was inserted slightly to pick a little of the bacteria.
6. Then the loop was streaked at the top end of the agar plate moving in a zig-zag horizontal pattern until $1/3^{rd}$ of the plate is covered.
7. Then the loop was again sterilized in the flame & cooled it at the edge of the agar away from the bacteria in the plate that just streaked.
8. The plate was rotated about 60 degree angle & was spread the bacteria from the end of the first streak into a second area by using the same motion in step 6.
9. Then again the loop was sterilized by using the procedure in step 7.
10. The plate was rotated about 60 degree & was spread the bacteria from the end of 2^{nd} streak into a new area in the same pattern & again the loop was sterilized.
11. Then the lid was replaced & the plate was kept in incubator overnight at 37 degree Celsius.

12. On the next day there were found bacterial cells growing along the streaks & isolated areas.
13. Next day there were seen circular, small to medium, thick, grayish white, moist, smooth, translucent colony of *E coli*.

Figure 3: Giving *E coli* streaking in blood agar

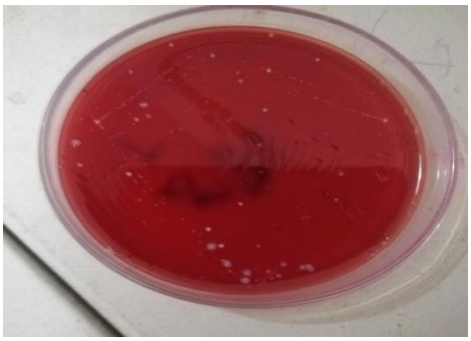

Figure 4: *Ecoli* colony in blood agar

Step 4: Brain heart infusion broth preparation

Equipments:

1. Brain heart infusion broth powder
2. Conical flask
3. Spatula.
4. Aluminum foil paper
5. Weighing balance
6. Distilled water
7. Autoclave
8. Test tube (2)

Procedure:

1. 2 gram of brain heart infusion broth powder was suspended in 50 ml of distilled water in a conical flask.
2. The mixture was then dissolved properly.
3. Then the mixture was pour into test tube & the test tube was kept in test tube rack.
4. Then these tubes were autoclaved at 121 degree Celsius for 15 minutes.
5. Then these tubes were kept in incubator overnight at 37 degree Celsius.

Figure 5: Brain heart infusion broth preparation

Step 5: *Ecoli* culture in BHI broth.

Equipments：

1. *E coli* culture
2. Brain heart infusion broth
3. Inoculating loop
4. Bunsen burner
5. Testube rack
6. Biosafety cabinet
7. Alcohol
8. Incubator

Procedure：

1. Before giving streaking biosafety cabinet area were disinfected through using 70% alcohol. Hands was also sterilized with alcohol. Inoculating loop was sterilized by placing it at an angle over a flame.
2. Then the loop was inserted slightly to pick a colony (*Ecoli*culture grow in blood agar) & scrape off a little of the bacteria using the loop.
3. The loop then inserted in broth& mixed gently& incubate it overnight at 37 degree Celsius.
4. The broth were turbid due to *Ecoli* growth.

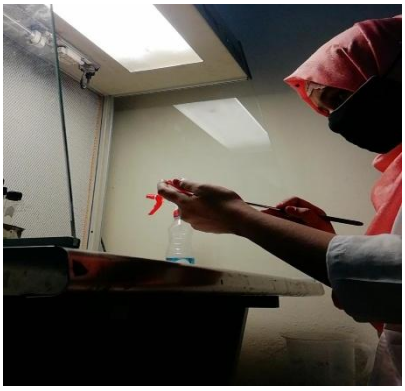
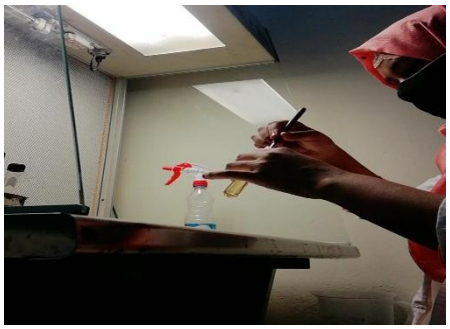


Figure 6:*E coli* culture in broth

Step 6: Trypton broth preparation.

Equipments:

1. Trypton broth powder
2. Conical flask
3. Spatula.
4. Aluminum foil paper
5. Weighing balance
6. Distilled water
7. Autoclave
8. Testube (10)

Procedure:

1. 2 gram of brain heart infusion broth powder was suspended in 100 ml of Distilled water in a conical flask.
2. The mixture was then dissolved properly.
3. Then the mixture was pour into testube & the testube were kept in testube rack. Each testube contain 9 ml of broth.
4. Then these tubes were autoclaved at 121 degree Celsius for 15 minutes.
5. Then these tubes were kept in incubator overnight at 37 degree Celsius.

Figure 7: Trypton broth preparation

Step 7:Serial Dilution of Phage Culture.

Equipments :

1. Trypton broth tubes.
2. Phage stock
3. 1000 microliter or 1 ml pipette
4. Micropipette
5. Marker
6. Alcohol

Procedure :

1. The biosafety cabinet area were disinfected through using 70% alcohol. Hands was also sterilized with alcohol. Inoculating loop was sterilized by placing it at an angle over a flame.
2. The trypton broth tubes that were kept in incubator on the previous day were marked from 10^{-1} to 10^{-10} with marker & kept in standing rack.
3. The cap of testube that contain phage culture were removed. The mouth of the tube then flamed in bunsen burner. Then 1 ml of phage culture was taken from the

phage stock tube, again flamed it mouth & capped it. The cap of trypton broth tube was also removed &phage culture that were taken into pipette was poured into the tube . Again capped the tube, mixed it well & replaced it in standing rack. The pipette tips was also discarded.

4. A new sterile pipette was taken. Then 1 ml of phage culture were taken from 10^{-1}tube, again recapped it & replaced it. The cap of 10^{-2} trypton broth tube were removed &those 1 ml culture were transferred into these tube, recapped it, mixed well & replaced it in standing rack.
5. The same procedure was repeated for the rest of the broth tubes leveled as 10^{-3} to 10^{-10}.Each time a new pipette was used.
6. After that, 1 ml of culture were removed from the 10^{-10} tube. Then again the tube were recapped.

Figure 8: Serial dilution of phage

Step 8:Muller hinton agar preparation.

Equipments :

1. Muller hinton agar powder
2. Conical flask
3. Spatula.
4. Aluminum foil paper
5. Weighing balance
6. Distilled water
7. Autoclave
8. Testube (5)

Procedure :

1. 4 gram of Muller hinton agar powder was suspended in 100 ml of distilled water.
2. The mixture was then to heated to dissolve all components properly.
3. Then the mixture was dispended into 5 testube. Each testube with 10 ml agar. The tubes were marked as 10^{-4} to 10^{-8}
4. Then the tube was autoclaved at 121 degree Celsius for 15 minutes.
5. After that all the tubes were kept in hot water bath at 100 degree Celsius for 5 minutes. After 5 minutes temperature were down at 45 degree Celsius till the completion of experiments.

Figure 9: Muller hinton agar preparation

Step 9:Inoculation of *E coli* & diluted phage culture in Muller hinton agar.

Equipments :

1. Micropipette
2. Pipette tips (100 microliter)
3. Agar plate
4. Alcohol

Procedure :

1. The biosafety cabinet area were disinfected through using 70% alcohol. Hands was also sterilized with alcohol. Inoculating loop was sterilized by placing it at an angle over a flame.
2. Muller hinton agar tube levelled as 10^{-4} were taken from water bath.
3. 100 microliter of *E coli* culture from BHI broth were taken through pipette & added into muller hinton agar tube that were levelled as 10^{-4}. Then recapped both the tube.
4. A new pipette was taken. Again 100 microliter of diluted phage culture were taken from trypton broth

that also levelled as 10^{-4}. Then transferred the phage into the 10^{-4} muller hinton tube.

5. Mixed properly & then gently poured into a agar plate levelled as 10^{-4}. The plate was closed by lid & gently shake to spread agar over.
6. The same procedure was repeated for all the other agar tubes that kept in water bath.
7. Then those plates were incubated in incubator overnight at 37 degree Celsius. On the next day the result was observed.

Figure 10: Inoculation of *E coli* & diluted phage culture in Muller Hinton Agar.

Results

I have seen that there were some opaque area has different color from surrounded area. Those are seems like plaques but after careful observation I have noticed that there was no hole of lysed cells in any plates. As there was no plaques formed, we can say that the protocol is not suitable for plaque count.

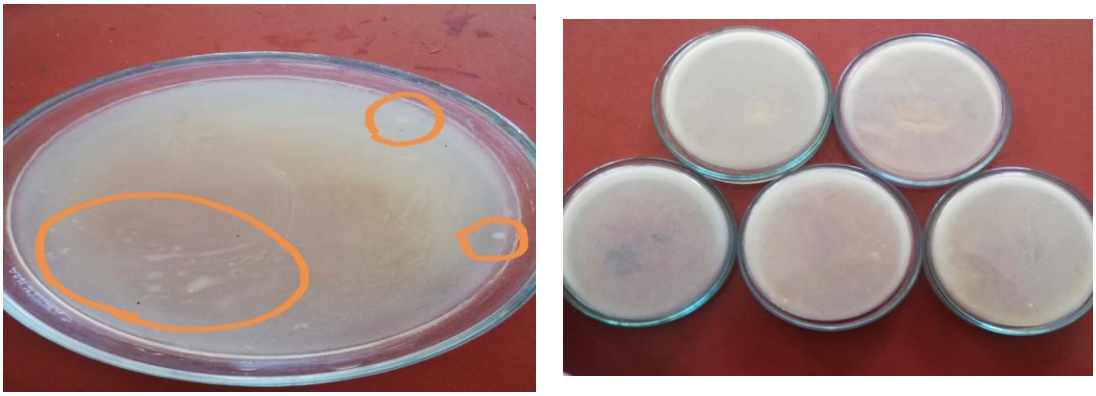

Figure 11: Plates after incubation (without plaques)

Discussion

In the current study, we tried to establish a protocol to count plaque of phages with our available laboratory products. In a video (https://youtu.be/IpL6Htcqo6I) there were used trypton broth for serial dilution of phages. Then 0.1 ml diluted phage & 0.3 ml *Ecoli* were inoculated in trypton soft agar & incubated overnight to see results. According to this video I have done serial dilution of phage in trypton broth. But as trypton soft agar was not available I have used Muller hinton agar instead of it. As there was no plaques observed in those plates then we can assume that there must be some factors that affect plaque formation by bacteriophages. As phages is strain specific we can assume that this *Ecoli* strain might not be specific for the phage. We were unable to use soft agar that can be also a reason of not getting plaques. Then again the phage culture we have used may not be of good quality. Therefore it can be said that this protocol cannot be used for plaque count of phages.

Conclusion

Bangladesh is a developing country. The economic development of our country much depend on livestock & poultry industry. Due to multi-drug resistance mortality of poultry are increasing day bay day which ultimately create a bad impact on economy of Bangladesh. Phage therapy as an alternative should be consider effectively as they have proven safe in therapeutic use &are rapidly adaptable to combat the bacterial infections. There is still need of further extensive research in the field of bacteriophages that will entail to discovery of new technique against rapidly increasing, resistant & mutant strains of bacteria.

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Biography

I am Jannatul Naima an intern student of the Faculty of Veterinary Medicine at Chattogram Veterinary and Animal Sciences University. I completed my Secondary School Certificate (SSC) and Higher Secondary Certificate (HSC) in 2014 and 2016 respectively from Chattogram. As a future veterinarian after completing my DVM degree, I would like to be a researcher and pursue higher studies in the field of public health and one health and contribute to the world with my knowledge and skills.