

# *In vitro* elution and *E. coli* and *T. pyogenes* killing profile of penicillin, ampicillin, tetracycline, tulathromycin, and florfenicol from Plaster of Paris beads

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## Research article

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# Abstract

**Background:** The objectives of this study were to create a protocol for making antibiotic impregnated Plaster of Paris (AI-PoP) beads using penicillin, ampicillin, tetracycline, tulathromycin, and florfenicol, to determine the in vitro elution rates of those antibiotics in the beads, and to quantify the in vitro inhibition of *E. coli* and *T. pyogenes* by the bead eluent in a proof-of-concept study using flow cytometry. The AI-PoP beads were made using Plaster of Paris powder, antibiotic, and tap water, cured for 24 hours, sterilized by ethylene oxide, and stored up to 5 months before testing. For each antibiotic, twenty beads were combined with bovine serum in sterile tubes and incubated at 37° C on a rocker. Serum was replaced at intervals over the 14 day study period, and antibiotic concentrations were determined by high pressure liquid chromatography with mass spectrometry. Separately, the growth of *E. coli* and *T. pyogenes* in eluent from 10 beads for each antibiotic was quantified by flow cytometry.

**Results:** Antibiotic was detected in AI-PoP bead eluent for 14 days for all but the ampicillin beads, for which antibiotic was detected for 8 days. The concentration of antibiotic in eluent was greater than five times the minimum inhibitory concentration (MIC) of tested bacteria for the entire study period for penicillin, tetracycline, and florfenicol. The concentration of ampicillin remained greater than five times the MIC of *E. coli* for 4 days and *T. pyogenes* for 5 days. The concentration of tulathromycin remained greater than five times the MIC of *E. coli* for 5 days and *T. pyogenes* for the entire study period. The CFU/ml of live *E. coli* and *T. pyogenes* was reduced over a 72-hour period by 1-3 log 10 CFU, with the exception of tetracycline, which reduced CFU/ml of *T. pyogenes* by less than log 10 CFU.

**Conclusions:** AI-PoP beads containing penicillin, tetracycline, tulathromycin and florfenicol elute antibiotic well above the MIC of tested bacteria for the two week study period and provide adequate local bacterial growth inhibition to reduce bacterial growth in vitro . These antibiotics show promise for delivery in joint and wound infections.

## Background

Synovial sepsis, post-operative surgical site infections, and complicated wounds are a devastating cause of increased morbidity and mortality in large animal veterinary patients and can be career or even life-ending.<sup>1-3</sup> While systemic antibiotics are the mainstay of treatment, complementary local antimicrobial therapies are often employed. These adjunct therapies are especially useful in the treatment of osteomyelitis, synovial sepsis, and surgical site infections.<sup>4-6</sup> The benefits of local therapy include the ability to achieve local drug concentrations many times above bacterial minimum inhibitory concentration (MIC), avoidance of high systemic doses of antibiotics, and cost effectiveness.<sup>7-9</sup>

The options for local antimicrobial therapy include intravenous and intraosseous regional limb perfusion<sup>10,11</sup> and sustained release of antimicrobials eluted from implanted carriers such as collagen sponges,<sup>12</sup> polymethylmethacrylate,<sup>13,14</sup> plaster of Paris (PoP),<sup>7-9</sup> constant rate infusion systems,<sup>15</sup> and hydroxyapatite cement.<sup>13</sup> Regional perfusions can achieve antimicrobial concentrations many times

higher than the MIC of common pathogens, however use is limited to the distal extremity due to the need to place a tourniquet proximal to the area of interest and because high concentrations of drug are generally not maintained for very long after tourniquet removal. Constant rate infusion systems are also able to achieve high antimicrobial concentrations, however breakage or blockage of the intrasynovial catheter is common.<sup>15</sup> Nonbiodegradable polymethylmethacrylate implants are easy to use, however removal may be necessary. In addition, creating the implant produces an exothermic reaction, thus only heat stable antibiotics may be used.<sup>15</sup> Biodegradable implants, including collagen sponges, hydroxyapatite cement, and PoP, offer the advantage of not requiring removal and being potentially more biocompatible. Purified type I collagen sponges are biocompatible, biodegradable, and are characterized by low immunogenicity.<sup>12</sup> Collagen sponges and hydroxyapatite cement have the disadvantage of being more expensive than PoP. Plaster of Paris, or calcium sulfate hemihydrate, is inexpensive, readily available, and easy to use for local antimicrobial delivery.<sup>15</sup> In addition, it is biocompatible, biodegradable, and osteoconductive when used in fracture repair and established osteomyelitis, providing a scaffold on which new bone formation can occur.<sup>7,15</sup> The *in vitro* elution of antibiotic from PoP beads has been reported for gentamicin, amikacin, clindamycin, enrofloxacin, vancomycin, tobramycin, and cefazolin.<sup>7-9,16,17</sup> These studies show that the elution profile is different among antibiotics.

The use of antibiotic impregnated PoP (AI-PoP) beads constitutes an extra-label drug use under the Animal Medicinal Drug Clarification Act of 1994, which also prohibits or limits the use of previously studied drugs in food producing species. Antibiotics elected for use in this study include penicillin, ampicillin, tetracycline, tulathromycin, and florfenicol. These antibiotics were chosen because they are commonly used in food animal practice and extra-label use may be allowed.

The objectives of this study were to (1) create a protocol for making AI-PoP beads using penicillin, ampicillin, tetracycline, tulathromycin, and florfenicol, (2) determine the *in vitro* elution properties of the AI-PoP beads over 14 days, and (3) quantify the *in vitro* inhibition of one isolate each of *E. coli* and *T. pyogenes* by the AI-PoP bead eluent over 72 hours.

We hypothesized that (1) the elution of all antibiotics from the AI-PoP beads would have a rapid initial phase and complete elution within two weeks, (2) the initial concentrations of antibiotic in the eluent would exceed the MIC of bacterial isolates used, and (3) AI-PoP bead eluent would inhibit the growth of single isolates of *E. coli* and *T. pyogenes* bacteria as determined by flow cytometry.

## Results

All AI-PoP beads eluted detectable concentrations of antibiotic for the 14-day sampling period, with the exception of ampicillin, which eluted detectable concentrations of antibiotic for 8 days. Mean observed peak ampicillin, florfenicol, and tetracycline concentrations occurred at four hours, mean observed peak tulathromycin concentration occurred at 12 hours, and mean observed peak penicillin concentration occurred at 24 hours (Figure 1, a-e).

The amount of antibiotic released in the first 72 hours, expressed as a percent of antibiotic released over the two-week study period, was greatest for ampicillin and least for florfenicol (Table 1). The total amount of antibiotic released over the two-week study period, expressed as a percent of total incorporated antibiotic, was greatest for florfenicol and least for tetracycline (Table 1).

The MIC data for each antibiotic and bacterial strain, as provided by the diagnostic lab at our institution, is shown in Table 2. Some authors consider time dependent antibiotics, such as those used in this study, to be most efficacious when concentration is maintained 2-5 times higher than the minimum inhibitory concentration (MIC) for at least 50% of the dosing interval.<sup>9</sup> The concentration of antibiotic within the eluent reported at each time point was greater than five times the MIC of both *E. coli* and *T. pyogenes* for penicillin, tetracycline, and florfenicol for the entire study period. The concentration of ampicillin remained greater than five times the MIC of *E. coli* for 4 days and *T. pyogenes* for 5 days. The concentration of tulathromycin remained greater than five times the MIC of *E. coli* for 5 days and *T. pyogenes* for the entire study period.

Ampicillin had the shortest elution half-life, and penicillin had the longest (Table 3). The elution half-life of florfenicol and tulathromycin could not be calculated in any of the samples because the slope was not negative.

The results of the flow cytometry proof-of-concept study are shown in Figure 2 a-b. The decrease in concentrations observed at 24 and 48 hours of growth represents the decrease in concentrations that resulted from adding 1 ml of Mueller Hinton (MH) broth to the culture tube following sampling. The CFU/ml of *E. coli* is reduced by approximately 1-2 log<sub>10</sub> CFU by 72 hours for all antibiotics compared to the control sample. The CFU/ml of *T. pyogenes* is reduced by 3 log<sub>10</sub> CFU for all antibiotics except tetracycline by 72 hours compared to the control sample. CFU reduction in the tetracycline sample was less than log<sub>10</sub> CFU.

## Discussion

This study was successful in creating antibiotic impregnated beads using five different antibiotics. The first hypothesis, that the elution of all antibiotics from the AI-PoP beads would have a rapid initial phase and complete elution within two weeks, was rejected. Only the elution of ampicillin was complete within two weeks; the remaining antibiotics eluted antibiotic for the full two-week study period. The second hypothesis, that the initial concentrations of antibiotic in the eluent would exceed the MIC of bacterial isolates used, was accepted. The concentration of antibiotic in the eluent exceeded five times the MIC of bacterial isolates for a minimum of four days. The third hypothesis, that the AI-PoP bead eluent would inhibit the growth of single isolates of *E. coli* and *T. pyogenes* bacteria as determined by flow cytometry, was also rejected. The growth of *T. pyogenes* did not appear to be inhibited by tetracycline; however, the growth of both bacteria was inhibited by the remaining antibiotics.

Size and composition of Al-PoP beads have previously been shown to influence elution pattern.<sup>18</sup> The amount of antibiotic eluted has been shown to be proportional to dissolution of the bead, with the rate of dissolution being affected by increases in porosity.<sup>19</sup> Porosity, determined by scanning electron microscopy,<sup>19</sup> and rate of bead dissolution were not analyzed in this study because the ideal porosity and other biomaterials properties for Al-PoP beads remains unknown. It has been speculated that factors such as storage temperature and humidity, length of time stored, and different antibiotics used may have an effect on bead dissolution.

This study is consistent with previous reports that found a variable volume of liquid is needed for individual batches in order to obtain an appropriate antibiotic-PoP mixture consistency prior to filling the bead mold.<sup>7,9</sup> Because of individual properties associated with each antibiotic, each Al-PoP bead preparation was different. Because procaine penicillin G was used, penicillin beads were cured and stored in a refrigerator in accordance with the handling instructions of the drug. It is unknown whether this was necessary, or if curing and storage at room temperature would have been acceptable.

A sampling period of two weeks was chosen in this study. Based on the rapid elution of antibiotic from Al-PoP beads observed in a previous study using PoP beads,<sup>7</sup> a similar elution profile was anticipated. However, we were surprised that in the current study, Al-PoP beads eluted antibiotic for the entire 14-day study period, with the exception of ampicillin. In previous studies using PoP beads, antibiotics such as gentamicin, amikacin, and clindamycin exhibited a rapid elution profile, while vancomycin, enrofloxacin, and tobramycin exhibited a prolonged release elution profile.<sup>7-9,16</sup> The elution pattern of penicillin, ampicillin, and tulathromycin in our study is similar to the previously reported elution pattern of most antibiotics,<sup>7,9</sup> characterized by an initial rapid release of antibiotic, subsequently followed by low concentration release for the remainder of the study period. The elution pattern of tetracycline and florfenicol is atypical in that a relatively constant level of elution was maintained for the entire study period. This pattern has previously been reported for enrofloxacin.<sup>9</sup>

Previous studies have used eluent to qualitatively describe bacterial inhibition.<sup>7,8</sup> Flow cytometry has been shown to be an accurate and rapid method of determination of the concentration of bacteria in a sample while distinguishing between live and dead organisms.<sup>20</sup> This proof-of-concept portion of the study serves to show that flow cytometry can be used to quantitatively describe the effect of Al-PoP eluent on bacterial inhibition.

The choice of antibiotics for systemic administration should be based upon culture and antimicrobial susceptibility data, but because of a variety of factors including cost, time to achieve C&S results, negative growth on cultures, and other reasons, the choice is often based upon clinical experience, empirical data, and commonly known bacterial isolates associated with specific conditions. The choice for local delivery of antibiotics is based upon similar criteria, although MIC breakpoints used to identify susceptible and resistant isolates are not predictive when antibiotic drugs are applied topically or locally, since achievable concentrations and effects on clinical outcome are unknown. Additional factors that

could potentially influence the performance of local antimicrobials include bacterial burden, wound contamination, and wound exudate. It remains unknown how this *in vitro* work is to relate *in vivo*. There are many factors of *in vivo* infection that were not replicated by this study, including microbe-related risk factors such as biofilm, and host-related factors such as an immune response.

The antibiotics chosen for this study were selected because they are labeled for use in food producing species and their extra-label use may be allowed. Food producing species are subject to restrictions on drug use such as provisions within the Animal Medicinal Drug Clarification Act of 1994. It is recommended that the Food Animal Residue Avoidance Database (FARAD) be consulted for any extra-label drug use, including those described in this report. It is unknown what the serum or tissue concentrations of these drugs might be after application of AI-PoP bead, so caution should be used when predicting withdrawal times.

A limitation of this study is that elution was only investigated for two weeks. Elution data showed that a longer period would have been beneficial for penicillin, tetracycline, tulathromycin, and florfenicol. An additional limitation of this study is that using PoP powder and water from sources that are not regulated for purity may serve as potential sources of variability on both the application of the suggested recipes, as well as the elution of antibiotic from AI-PoP beads. Another limitation is that the materials properties of the beads were not analyzed, thus we were not able to provide further information regarding the rate of dissolution of the beads.

## Conclusions

Future studies should focus on the *in vivo* application of ampicillin, florfenicol, penicillin, and tulathromycin AI-PoP beads to corroborate the results of this *in vitro* study. Ampicillin, florfenicol, penicillin, and tulathromycin appear to perform well in eluting antibiotic from AI-PoP beads and inhibiting the growth of *E. coli* and *T. pyogenes*. These antibiotics show promise for use AI-PoP beads in food animal species.

## Methods

Based on methods previously described,<sup>7,9</sup> AI-PoP beads were made as follows: twenty grams of PoP powder was thoroughly mixed with antibiotic powder or liquid in a specimen cup. When needed, water was added to achieve a pourable mixture (see Table 4 for weights and volumes used). Ampicillin and tetracycline powder were used, whereas penicillin, tulathromycin, and florfenicol were in solution. All AI-PoP bead ingredients were chosen for being inexpensive and easily accessible to general practice veterinarians, as it was desired to create a bead recipe that could be replicated and used in a general practice setting. In this study, all PoP powder used was derived from the same carton, all water was obtained from the same source in the hospital, and all antibiotics were derived from the same bottle or vial. All bead types used experimentally were created in a single batch, thus eliminating the need to control for variability in lot numbers. Each mixture was poured into a silicone candy bead mold (Chicago

Culinary FX, Westchester, IL) and a wooden tongue depressor was used to completely fill the wells. The use of a silicone bead mold allowed standardization of bead size in this study. The AI-PoP beads were cured for at least 24 hours at room temperature (with the exception of penicillin beads, which were cured at 4 °C), sterilized by ethylene oxide, and stored at room temperature (with the exception of penicillin beads, which were stored at 4 °C based on storage conditions identified on the drug label). Testing was completed within five months of AI-PoP bead manufacturing.

For each antibiotic, twenty AI-PoP beads and seven milliliters of bovine serum (Lampire Biological Laboratories, Inc., Pipersville, PA) were aseptically transferred to individual sterile polypropylene test tubes. The number of beads, choice of serum, and volume of serum were chosen based on previous studies<sup>7,9</sup> and because it was sufficient to immerse the beads in serum. The tubes were maintained in a rotating incubator at 37 °C, except when removing eluent and adding serum. The entire volume of eluent was removed at specified time points and replaced with fresh bovine serum (Table 5). As previously described,<sup>7</sup> the volume of serum was decreased to 5 mL at four days to reflect the decrease in effusion commonly observed in clinical patients. Eluent samples were stored at -70 °C until analyzed (approximately one month). The eluent from each AI-PoP bead type was tested in quadruplicate; four test tubes of AI-PoP beads were tested for each antibiotic.

Concentrations of five antibiotics were determined using high-pressure liquid chromatography with mass spectrometry detection (LC-MS/MS) after protein precipitation of samples with acetonitrile. LC-MS/MS was performed using an Agilent 1100 Pump, column compartment, and autosampler (Santa Clara, CA, USA) coupled to an ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA, USA). Serum samples, serum spikes, serum quality controls, and bovine serum blanks, 50 or 100 µL, were mixed with 450 or 400 µL of acetonitrile to precipitate serum proteins. The acetonitrile contained an internal standard at a concentration of either 250 or 1000 ng/mL. The samples were vortexed for 5 seconds and centrifuged for 10 minutes at 7500 rpm to sediment the protein pellet. A portion, 20 or 50 µL, of the supernatant was then diluted with water to 1 mL of volume in an autosampler vial. Further dilution of the samples, if needed, was done with blank serum that had been precipitated and diluted identically as the samples. These dilutions were done in autosampler vials fitted with glass inserts and dilutions were 1:5 or 1:10. The vials were then centrifuged at 2,400 rpm prior to analysis. All the quality control samples passed the tolerance of being within  $\pm 15\%$  of the nominal concentration. The limit of quantitation of the analysis was 0.5 µg/mL with a limit of detection of 0.1 µg/mL.

The antimicrobial activity of eluent was evaluated against two bacterial isolates, an American Type Culture Collection (ATCC) strain of *E. coli* and a clinical isolate of *T. pyogenes*. These are two of the most common isolates in cattle from cases of synovial sepsis.<sup>21</sup> Together they encompass a broad spectrum of bacteria, *T. pyogenes* being a slow growing Gram-positive facultative anaerobe, and *E. coli* being a fastidious Gram-negative facultative anaerobe.<sup>22,23</sup> Using the McFarland standard, colonies of pure cultures of *E. coli* and *T. pyogenes* were used to inoculate test tubes containing ten AI-PoP beads and five milliliters of MH broth to a concentration of  $10^7$  CFU/mL. This concentration was elected because it was

the most concentrated inoculum that could be reasonably achieved using these methods. It has been shown that the risk for surgical site infection is markedly increased when contaminated with more than  $10^5$  microorganisms per gram of tissue.<sup>24</sup> Thus, the inoculation dose of  $10^7$  was considered sufficient to represent clinical infection. A single negative control test of MH broth lacking AI-PoP beads was also inoculated at the same concentration for each bacterium. Following inoculation, the tubes were maintained in a rotating incubator at 37 °C. Twenty-four, 48, and 72 hours after inoculation, a one milliliter sample of eluent was obtained for quantification of live and dead bacteria by flow cytometry, and the volume was replaced with MH broth. In this proof-of-concept phase of the study, a single test of each AI-PoP and bacteria combination was completed. Live and dead bacteria in samples were stained for quantification by flow cytometry using a commercially available kit (Molecular Probes, Inc.; Eugene, OR). Samples were prepared according to manufacturer's instruction, then immediately transported to the flow cytometry laboratory for quantification. The number of events in the bead region was set to 5,000 and the sample was assayed at 488 nm. The data were processed by framing the region around the live and dead populations to yield events data for live and dead bacteria. The bacterial culture density was calculated using outcome events data and dilution factors.

The elution half-life was calculated based on the log-linear regression of the terminal portion of the concentration-time curve using standard pharmacokinetic software (Certera Phoenix 64 8.1.0.3530).

## List Of Abbreviations

AI-PoP: antibiotic impregnated Plaster of Paris

MIC: minimum inhibitory concentration

PoP: Plaster of Paris

MH: Mueller Hinton

FARAD: Food Animal Residue Avoidance Database

LC-MS/MS: high-pressure liquid chromatography with mass spectrometry detection

ATCC: American Type Culture Collection

## Declarations

*Ethics approval and consent to participate*

Not applicable

*Consent for publication*

Not applicable

### *Availability of data and materials*

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### *Competing interests*

The authors declare that they have no competing interests.

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### *Authors' contributions*

PM, JS, AK, and DB contributed to the design and completion of this study. All authors contributed to the writing, editing, and final approval of the manuscript.

### *Acknowledgements*

Not applicable

### *Authors' information*

Not applicable

### *Footnotes*

Not applicable

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## Tables

	14d/ total	72h/ 14 d
Ampicillin	45.23	99.88
Florfenicol	49.23	58.43
Penicillin	33.55	67.62
Tetracycline	3.79	59.45
Tulathromycin	16.83	90.02

Table 1. Percent elution in terms of total included antibiotic and antibiotic eluted over the 14-day study period.

	E. coli	T. pyogenes
Ampicillin	4.0000	0.2500
Florfenicol	4.0000	1.0000
Tetracycline	1.0000	4.0000
Penicillin	>8.0000	<=0.1200
Tulathromycin	8.0000	<=1.0000

Table 2. Minimum inhibitory concentration of isolates used.

	Rep 1	Rep 2	Rep 3	Rep 4	Mean
Ampicillin	8.8	9.4	12.0	8.5	9.6
Penicillin	248	236	185	216	221.2
Tulathromycin	101	101	124	95	105.4
Florfenicol	Could not be calculated because slope was not negative				
Tetracycline	Could not be calculated because slope was not negative				

Table 3. Elution half-life (in hours) of antibiotics in AI-PoP beads incubated for 14 days in bovine serum.

	Penicillin	Tetracycline	Ampicillin	Tulathromycin	Florfenicol
PoP powder (g)	20	20	20	20	20
Antibiotic	12 ml	2000 mg	2000 mg	3 ml	3 ml
Water (ml)	0	8	9	5	6
Total antibiotic (mg)	2257	2000	2000	300	900
Yield (beads)	132	134	121	108	116
Bead concentration (mg/bead)	17.1	14.9	16.5	2.78	7.76
Total Abx Tested (mg)	1368	1192	1320	222.4	620.8

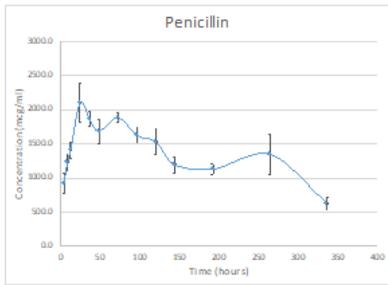
Table 4. Antibiotic impregnated Plaster of Paris bead recipes

Time (hours)	Serum (mL)
4	7
8	7
12	7
24	7
36	7
48	7
72	7
96	5
120	5
144	5
192	5
264	5
336	5

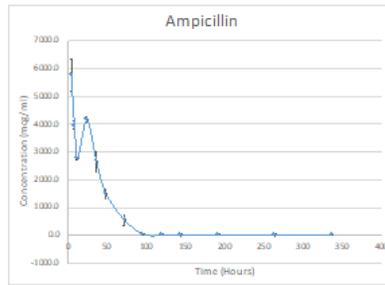
Table 5. Time in hours that given volume of serum was removed.

## Figures

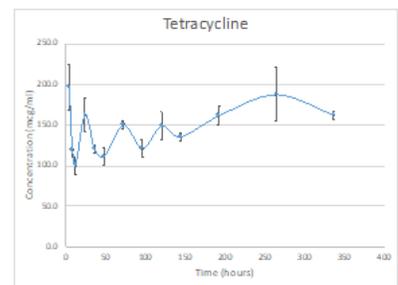
**Figure 1. The concentration in mcg/ml is shown over the two week study period for each antibiotic.**



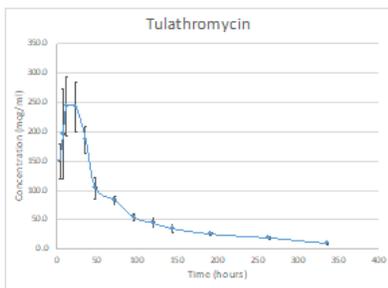
**a. Penicillin**



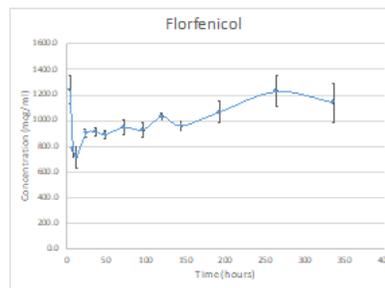
**b. Ampicillin**



**c. Tetracycline**



**d. Tulathromycin**

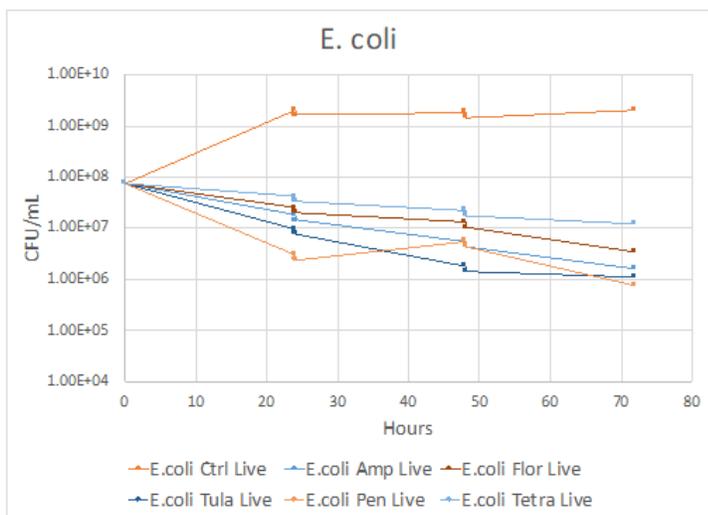


**e. Florfenicol**

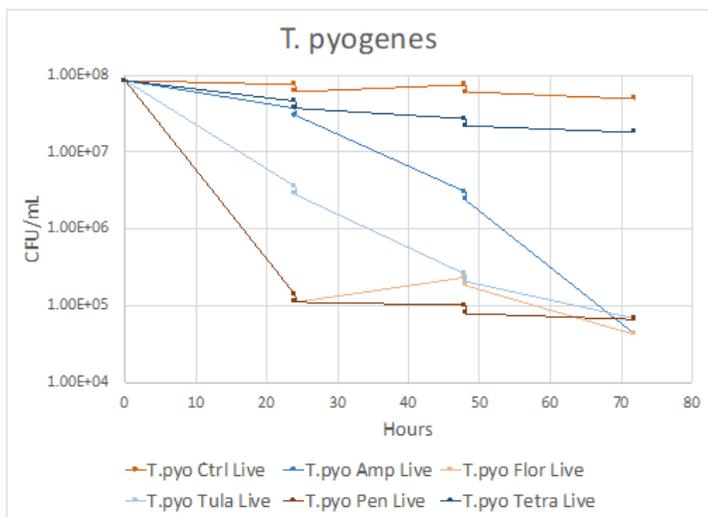
**Figure 1**

[See figure]

Figure 2. The CFU/ml of each antibiotic and the control at 24, 48, and 72 hours of growth.



a. *E. coli*



b. *T. pyogenes*

Figure 2

[See figure]