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# Evaluation of Antibiotic Activity of Tetracycline on Lactobacillus acidophilus

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## ABSTRACT

The zone of inhibition test can be used to measure the susceptibility of the bacteria towards the antibiotic.The process of measuring the diameter of this Zone of Inhibition can be automated using Image processing or can be measured manually. The aim of this work is to evaluate the antibiotic activity of Tetracycline on **Lactobacillus acidophilus**. This work demonstrates an effective approach of evaluating the Zone of Inhibition by calculating the radius of the zone by drawing contours and setting the right value of threshold. This work also determines if a particular bacteria is susceptible or resistant to the applied antibiotic using the calculated Zone of Inhibition and the prescribed standard values.This work also demonstrate the need of additional probiotics therapy during the course of Tetracycline therapy.

**Key words:-**Spices, Antimicrobial activity, Inhibition, Organic compound.

## I. INTRODUCTION

**S**ensitivity or resistance of microorganisms to known or potential antimicrobial compounds is most often determined by the zone of inhibition test, also known as disk diffusion test or Kirby–Bauer test[1,2]. The test is widely used and helps physicians decide whether or not to prescribe a certain antibiotic for an infected patient. The procedure for performing the test has been standardized by the World Health Organization. [3]. In brief, cells are spread on a plate and a small filter paper disk impregnated with the antibiotic is placed in the center of the plate. The absence of growth around the disk indicates sensitivity to the antibiotic. The diameter of the zone of inhibition is only a qualitative indicator of the potency of the drug as there are several other factors involved such as depth of the agar as well as the size and water solubility of the drug molecule. Nevertheless, the method is an easier and cheaper alternative than the broth dilution or the agar dilution methods. Characterization of new antibiotics usually includes a zone of inhibition result[4].

Aggregatibacteractinomycetemcomitans, a naturally transformable, [5] Gram-negative facultative anaerobe, is a periodontal pathogen[6]that also belongs to the HACEK group of bacteria, which is known to cause endocarditis[7]. It has been observed in our laboratory that the zone of inhibition for A. actinomycetemcomitans has been difficult to detect because the colonies have a similar color as the background agar, resulting in the confluent colonies appearing transparent. Even if a faint zone of inhibition can be visualized by adjusting the intensity and angle of light source, photodocumentation of the faint zone of inhibition is very difficult. Other bacteria such as E. coli and A. vinelandii have also been reported to have transparent colonies and are difficult to photograph unless the incident light is carefully adjusted[8]. In this paper I show that this problem can be solved if the background agar and the microbial colonies can be stained differently.

Agar is a complex mixture of two carbohydrate polymers: agarose, a neutral linear polymer containing alternating units of β-1,3-linked- D-galactose and α-1,4-linked 3,6-anhydro-L-galactose; and agaropectin, an anionic polymer containing the same repeating unit as agaroseexcept that some of the galactose units are modified with negatively charged side groups such as sulfate[9]. As agaropectin is negatively charged, it can be expected to bind cationic dyes such as crystal violet and methylene blue. Although, bacterial cell walls also bind cationic dyes, the stained cells can be expected to be differentiated from stained agar because of a metachromatic property of the dyes. Binding of crystal violet or methylene blue to anionic polysaccharides such as

agaropectin results in a shift in λmax[10]. For example, λmax of crystal violet shifts from 585 to 510 nm. The shift is ascribed to 'stacking interactions' as excess dye molecules adsorb to dye molecules initially bound by electrostatic interactions[11].So, staining of the agar plate with cationic dyes will stain the cells and the zone of inhibition differently.

There are several experiment where it have been foundthat staining plates with crystal violet or methylene blue, the zone of inhibition can be not only more clearly visible but also easily photographed. Barely visible zone of inhibition is a common problem with many microorganisms. Improvements were found in visibility which was achieved for the Gram-negative bacteria, **A. Actinomycetemcomitans** (CU1000, a rough clinical isolate12), Escherichia coli (MV10) and Proteus vulgaris (ATCC 6380); and the Gram-positive bacteria, **Enterococcus faecalis** (ATCC 51299) as well as for yeast, **Saccharomyces cerevisiae** (baker's yeast, Fleischmann). The method can be applied universally for all microorganisms even if the zone of inhibition is already clearly visible. Gram-positive bacterium, Staphylococcus aureus (ATCC 25923) has been used as an example to demonstrate this.

Zones of inhibition are barely visible for **E. coli** and **P. vulgaris** and almostinvisible for **A. actinomycetemcomitans** and **S. cerevisiae**. However, the zones are clearly visible after staining with either crystal violet or methylene blue. Methylene blue gives slightly better results than crystal violet. Results with a rough clinical isolate of **A. actinomycetemcomitans** (CU1000) are shown here. A commonly used smooth laboratory variant (Y4) of a clinical isolate is even more transparent and more difficult to visualize unless stained with crystal violet or methylene blue (data not shown). The zones of inhibition for the Gram-positive bacteria, **E. faecalis** and **S. aureus**, especially the latter are clearly visible even without staining. However, it is a good idea to apply the staining procedure for all bacteria, as the zone will be even more clearly visible and also some important features may be visible only after staining. This is seen for **S. aureus** after staining with methylene blue; a layer of yellow colour is present at the boundary of the zone of inhibition. However, such extra ring is not clearly visible without staining the plate. The yellow colour is indicative of a higher cell density as dense regions are destained more than isolated colonies. It has been reported before that there can be a greater cell density at the boundary of a zone of inhibition, which happens because of greater amount of nutrients available[12]. Similar to zone of inhibition studies, the Kirby–Bauer disk diffusion method can also be used to study growth promoting effect of some compounds[13]. Staining with methylene blue can probably be used to visualize regions of extra growth in such experiment.

Being a cationic dye, crystal violet can be used to stain negatively charged polymers such as DNA18 and cell walls of bacteria, the latter of which is partially the basis of Gram staining to differentiate between Gram-positive and Gram-negative bacteria. It was shown that actually isolated cell wall material from Gram positive and Gram-negative bacteria binds crystal violet to the same extent[14]. The differentiation between the two types of bacteria is due to the subsequent steps of the Gram-staining procedure. In this report, I used only crystal violet and not the subsequent reagents of Gram-staining. This method can be used for both Gram-positive and Gram-negative bacteria. Destaining of cells was done with 50% ethanol. Even after five washes, more crystal violet can be washed out but that is not recommended because the background colour of the agar will diminish. While further washings, the isolated colonies will remain coloured but the dense region will be destained more (data not shown). On the other hand, methylene blue stain on background agar is very stable to further washings. If the filter paper disk comes off during destaining, it can be carefully placed back in the center or it may be completely removed; in case of the latter the region in the center will appear as unstained or less stained area.

The zone of inhibition method is qualitative and can only be used to classify the microbial strains as being susceptible, intermediate or resistant. The concentration of the antibiotic at the boundary of the zone of inhibition is the MIC of the antibiotic; however, it is not feasible to measure that concentration. A better and easier method for determining the MIC is the E-test in which a plastic strip containing a gradually decreasing concentration of an antibiotic is placed on an agar plate. Transparent and translucent colonies that are difficult to visualize and photograph will present a similar problem for the E-test and thus, the problem can also be expected to be solved by differentially staining agar and the cells with crystal violet or methylene blue. Observation of a zone of inhibition can also be used as a method for isolating new antibiotic-producing microorganisms[15]. It can be expected that staining with cationic dyes will also help in that procedure.

Here we use the curd for culturing the **Lactobacillusacidophilus**. It is considered useful for human health purposes because it doesn't cause

disease. It also produces vitamin K and lactase, the enzyme that breaks down the sugars in milk products[16].

## II. MATERIALS AND METHODS

1. **Preparation of Agar Media:-** At first clean the glasswares properly with water, then rinse them with distilled water. Then put the glasswares in the hot air oven and dry them.Place the dry petri plates in the laminar air flow in the aseptic condition .Then rinse them with ethanol and let them dry. And sterilize them by UV sterilization.Other hand take 500ml water in a 1L measuring cylinder. Then pour a little much water in the conical flask. Add beef extract in the conical flask and shake it anti clock wise or clock wise until it dissolved.Then mix the peptone powder into it and shake anti clock wise or clock wise. Then add some water and add the agar powder and repeat the process. Then add NaCl and mix. Then make up the volume upto 500ml and plug it with cotton and wrap it with aluminium foil , and place it on the hot plate , heat it until the bubbles are formed. Then place the conical flask into the autoclave for 1 hour in 121° C for sterilization. After this process take the conical flask into the laminar air flowby maintaining the aseptic condition. Maintaining the aseptic condition, pour the nutrient agar media in the petri plates, and let them cool down. After the procedure put the petri plates into the incubator and maintaining the temperature 37° C or 98.6°F for 24 hours to see any contamination.
2. **Isolation of Lactobacillus acidophilus from curd:** Traditionally Curd is well known for the source of lactobacillus spp. 10gm Curd was transferred in to a sterilized flask. Then it was diluted serially from $10^{-1}$ to $10^{-14}$ from this 14 dilutions $10^{-5}$ $10^{-7}$ and $10^{-9}$ were taken. Then they were incubated at $37^0$C which considered as optimum temperature for growth lactobacillus species. After 24 hours 3 isolated colonies were found .After Colony characterization those colonies found to be lactobacillus species. Among them 1 colony shows 100% resemblance with lactobacillus acidophilus. Identification test was done according to Bergey's manual[17].
3. **Culture the Lactobacillus acidophilusby using curd:-**First wash the glasswares properly with water and then rinse them with distilled water and place them into the laminar air flow, and sterile them with UV sterilization. Then place the isolated **Lactobacillus acidophilus** and previously prepared agar media plate in the laminar air flow by maintaining the aseptic condition.Add little much of isolated **Lactobacillus acidophilus** into the beaker, add some amount of water then stir it properly.Then measure 1ml of curd water and add it into the nutrient media. Then place them into the incubator and setting the temperature at 37°C for 24 hours for growth of bacteria.
4. **Preparation of Nutrients Broth and Culture the Bacteria:-**Clean the glasswares with clean water, then rinse them with Distilled water. Add little much of water in the beaker then add the dry ingredients into it and shake it anti clock wise or clock wise and make up the volume. Then heat it through a hot plate up to 50-55°C. And plug a cotton plug onto it.Maintaining the aseptic condition transfer it to the laminar air flow.Heat sterilize the inoculation loop by the Bunsen burner, then take the bacteria sample from the previously prepared bacterial culture.After taking the bacteria sample mix it into the nutrient broth. Measure 1ml of nutrient broth, add it into the previously prepared culture media plate.Place it into the incubator maintaining the temperature 270° for 24 hours.
5. **Zone Inhibition Test for Lactobacillus acidophilus by using Tetracycline250mg**:- Weigh the tetracycline powders accurately. Wash the glasswares with clean water.Take 50 ml of water in a beaker, add 2190mg of powder and stir it with a help of glass rod for 30 minutes.After the proper mixing, filter it through the filter paper, it is the stock solution of tetracycline with 40mg/ml concentration.Take 25ml of stock solution and dilute it with 25 ml of water, its concentration is 20mg/ml.Take 3.3 ml of solution from the stock solution and dilute it with 6.7ml of water and here the concentration is 13.2mg/ml.Take 25 ml of solution from 20mg/ml solution and dilute it with 25ml of water. And here the concentration is 10mg/ml. Take some punch paper and drop them into four different solution and let it for 1 hours for soaking.After 1 hour take them into laminar air flow. Then take a punch paper from $\frac{1}{25}$ concentrated solution by heat sterilized pinching tool and place it a side of previously prepared bacterial culture plate, then take another punch paper from $\frac{1}{50}$ concentrated solution and place it another side of same petri plate. Then seal it by parafine paper and marking it. Repeat the same procedure for $\frac{1}{75}$ and $\frac{1}{100}$ .Then keep the petri plates into the incubator for 24 hours to 48 hours and maintaining the temperature at $37^0$C.

**Figure 01: ZOI of Tetracycline** 40mg/ml&20mg/ml on Lactobacillus acidophilus

**Figure 02: ZOI of Tetracycline** 31.2mg/ml&10mg/mlon Lactobacillus acidophilus

## III. RESULT & DISCUSSIONS

Here we weigh the powder of tetracycline capsules. As per requirements we weigh 10 capsules by using electronic weighing device.

Table :01

| Sl No. | Capsule | Strength |
|---|---|---|
| 1 | Cap 1 | 270 mg |
| 2 | Cap 2 | 290 mg |
| 3 | Cap 3 | 260 mg |
| 4 | Cap 4 | 270 mg |
| 5 | Cap 5 | 280 mg |
| 6 | Cap 6 | 270 mg |
| 7 | Cap 7 | 270 mg |
| 8 | Cap 8 | 270 mg |
| 9 | Cap 9 | 280 mg |
| 10 | Cap 10 | 280 mg |

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Total weight of capsule powder 2740mg
The average weight= 2740/10 = 274
250mg drug present in274mg of powder
So, 100 mg drug present in 274/250*100=109.6
So, 1000 mg drug present in 1096mg of powder.
And 2000 mg drug present in 2192mg of powder.
Here we make the four different drug concentrations like,
$\frac{1}{25}, \frac{1}{50}, \frac{1}{75}, \frac{1}{100}$
For $\frac{1}{25}$ concentration,
For 25 ml solution here we dissolve 2000 gm of drug, it means we need 2192mg of powder dissolves in 25 ml of water. The drug concentration is 2000/25=40mg/ml. This is the stock solution.
After 24 hrs for this concentration the diameter of zone of inhibition was found 26mm.

For $\frac{1}{50}$ concentration,
For this concentration we take 25ml of stock solution and dilute it in with 25ml of water. Its become 1000/50= 20mg/ml.

After 24 hrs for this concentration the diameter of zone of inhibition was found 21 mm.

For $\frac{1}{75}$ concentration,
For making this concentration we take 3.3ml solution from stock solution and dilute it with6.7ml of water. It becomes 13.2mg/ml.
After 24 hrs for this concentration the diameter of zone of inhibition was found 17 mm.

For $\frac{1}{100}$ concentration,
For making this concentration we take 25ml of solution from$\frac{1}{50}$ and dilute it with 25ml of water. And its become 500/50=10mg/ml.
After 24 hrs for this concentration the diameter of zone of inhibition was found 08 mm.

## IV. CONCLUSION

The zone of inhibition is very key aspects for determination of the effectiveness of any antibiotics. This particular study also correlates the necessity of additional probiotics therapy during the treatment of any kind of infection with tetracycline. **Lactobacillus acidophilus** has several health benefits for digestion and this study clearly indicates that administration of tetracycline will cause destruction of probiotics and strongly indicates the necessity of simultaneous probiotics administration with tetracycline, as milk and milk products are not recommended with tetracycline therapy which are the main source of Lactobacilli species.

**Declaration**
**Authors declare no conflict of interest.**

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