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Antibiotic sensitivity of *E.coli.* for chloramphenicol by using Mueller Hinton Agar media and Kirby-Bauer test.

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Abstract – E.Coli. is sensitive for antibiotic chloramphenicol; continuous exposure of chloramphenicol to E.Coli. develops certain resistant so that sensitivity to antibiotics chloramphenicol decreases over the time. Immunity against chloramphenicol may generated because of random mutations in E. Coli. Genome as a result chloramphenicol become unable to interfere with protein synthesis mechanism of E. Coli. Study the antibiotic sensitivity of *E.coli.* for chloramphenicol by using Mueller Hinton Agar media and Kirby-Bauer test and the antibiotic resistance to chloramphenicol for *E.coli.*

Keywords – Chloramphenicol, antibiotics, *E. coli*

Introduction

The word chemotherapy can be defined as the use of chemical compounds in the treatment of infectious diseases, so as to destroy offending organisms' parasites without damaging the host tissues.The evolution of chemotherapy can be traced through three distinct periods:

- A pre-Ehrlich era before 1891.
- Period of Paul Ehrlich; and
- Period after 1935 highlighted by the discovery of sulfonamide and antibiotics.

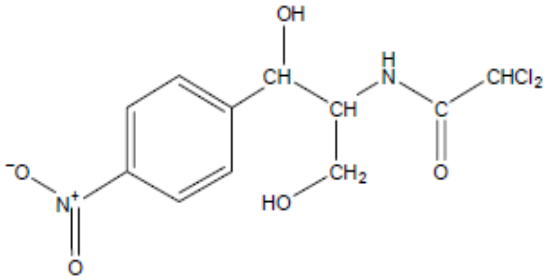
Antibiotics are the chemical substance produced by microorganisms having the property of inhibiting the growth of or destroying other micro-organisms in high dilution. The chemotherapeutic agent may act by destroying the organism (**bactericidal)** or by inhibiting its growth (**bacteriostatic).** The selective toxic action on the infecting organism is the key to beneficial actions on the infecting organism is the key to beneficial actions of antibiotics. Chloramphenicol is a broad-spectrum antibiotic derived originally from streptomyces *Venezuela in 1947.*The commercially available drug, however, is now entirely synthetic.
Chloramphenicol is a derivative of dichloroacetic acid and contains a nitrobenzene moiety. It was soon synthesized chemically and the commercial product now is all synthetic.

Physical-chemical properties

- ❖ Chloramphenicol exists as a white to grayish-white or yellowish-white fine crystalline Powder, needles, or elongated plates.
- ❖ Melting point 150.5 to 151.5^{O}C.
- ❖ The antibiotic is stable over the pH range of 2 to 9.

- It sublimes in high vacuum and is sensitive to light.
- Aqueous solution is quite stable, stands boiling.
- The nitro group is readily reduced to the amine group.
- The four possible stereoisomers.
- Only the αR, βR (or D-*throe*) form is active (IARC 1990).
- It has a nitrobenzene substitution, which is probably responsible for its antibacterial activity and its intensely bitter taste.

Structure of Chloramphenicol



Property	Information	Reference
Molecular weight	323.1322	Budavari *et al.* 1996, ChemFinder 2000
Color	white to grayish-white or yellowish-white	Budavari *et al.* 1996, CRC 1998, ChemFinder 2000
Taste	bitter burning	HSDB 1995
Physical state	crystals, crystalline powder, needles, or elongated plates	Budavari *et al.* 1996, CRC 1998, ChemFinder 2000
Melting point (°C)	150.5–151.5	Budavari *et al.* 1996, CRC 1998, HSDB 1995
pH	neutral to litmus	HSDB 1995
Vapor pressure (mm Hg)	1.73×10^{-12}	HSDB 1995
Half-life in humans	1.6–4.6 h	HSDB 1995
Solubility		
Water at 25°C	slightly soluble, 2.5 mg/mL	ChemFinder 2000, HSDB 1995
Propylene glycol	150.8 mg/mL	HSDB 1995
50% Acetamide	5%	HSDB 1995
Chloroform	soluble	HSDB 1995
Methanol	very soluble	HSDB 1995
Ethanol	very soluble	HSDB 1995
Butanol	very soluble	HSDB 1995
Ethyl acetate	very soluble	HSDB 1995
Acetone	very soluble	HSDB 1995
Ether	soluble	HSDB 1995
Benzene	insoluble	HSDB 1995
Petroleum ether	insoluble	HSDB 1995
Vegetable oils	insoluble	HSDB 1995

Review of Literature

Mechanism of action

Chloramphenicol inhibits bacterial protein synthesis by interfering with "transfer of the elongating peptide chain to the newly attached aminoacyl –t RNA at the ribosome-mRNA complex. It specially attaches to the 50S ribosome and thus may hinder the access of aminoacyl-tRNA to the acceptor site for amino acid incorporation.

Antimicrobial activity

The antibacterial spectrum of chloramphenicol resembles that of chloramphenicols. Chloramphenicol is primarily bacteriostatic. It is a broad-spectrum antibiotic, active against the same range of organisms (gram positive and gram negative, Rickettsiae, Chlamydia, Mycoplasma) as chloramphenicol.

Notable difference between these two are-

- Chloramphenicol is highly active against Salmonella including S.typhi, but resistant strains are now rampant.
- It is more active than chloramphenicol against H.influenza (through many have now developed resistance), B.pertussis, Klebsiella and anaerobes including Bact. fragilis.
- It is less active against gram positive cocci, spirochetes, certain enterobacteria, inactive on Entamoeba and Plasmodia.

- Like tetracycline, it is inactive against Mycobacteria, Pseudomonas, many Proteus, viruses and fungi.
- Chloramphenicol is less active against gram positive cocci than penicillin or chloramphenicol; essentially it is bacteriostatic drug but can be bactericidal against comment meningeal pathogens – *H.Influenzae, N.Meningitidis and S. Pneumoniae.*

The chloramphenicol is primarily bacteriostatic, through high concentrations have been shown to exert cidal effect on some bacteria. e.g., H. influenza. It is broad spectrum antibiotic, active against the same range of organisms.

Its antimicrobial spectrum of chloramphenicol resembles that of chloramphenicol. Thus, it is effective against Rickettsia, the chlamydia of the psittacosis lymphogrnuloma group, Mycoplasma pneumoniae, and against a variety of Gram-positive and Gram-negative organisms. Salm. typhi, H.influenza and H.pertussis are more susceptible to chloramphenicol than to almost any other antibiotic. The other Gram-negative organisms sensitive to chloramphenicol include Shigella, E.coli, K pneumoniae, A. aerogens certain strains of Proteus, Pasturella, Brucella and Vibrio. At high doses it can inhibit mammalian mitochondrial protein synthesis as well. Bone marrow cells are especially susceptible. Resistance develops slowly to these antibiotics in vivo. However, resistant strains of E.coli salmonella, shigella and other gram-negative bacteria have been reported ; it is due to the presence of a specific resistance (R) factor. E. coli may exhibit a cross resistance to chloramphenicol and chloramphenicol's.

Resistance

Most bacteria are capable of developing resistance to chloramphenicol, which generally emerges in a graded manner, as with chloramphenicol. Being orally active, broad spectrum and relative cheap, chloramphenicol has been extensively and often indiscriminately used, especially in developing countries, resulting in high incidence of resistance among many gram-positive and gram-negative bacteria.

In many areas, highly chloramphenicol resistance S.typhi have emerged due to transfer of R factor by conjugation. Resistance among gram negative bacteria is generally due to acquisition of R-plasmid encoded for an acetyl transferase –an enzyme which inactivates chloramphenicol. Acetyl chloramphenicol does not bind the bacterial ribosomes. Decreased permeability into the resistant bacterial cells (chloramphenicol appears to enter bacterial cell both by passive as well as facilitated diffusion) and lowered affinity of bacterial ribosomes for chloramphenicol are other mechanisms of resistance.

Pharmacokinetices

Chloramphenicol is rapidly and completely absorbed after oral ingestion. It is 50-60% bound to plasma proteins and very widely distributed: volume of distrubution 1L/kg.It freely penetrates serous cavites and blood brain barrier :CSF concentration is nearly equal to that of unbound drug in plasma .It crosses plancenta and is secreted in bile and milk.

Chloramphenicol is primarily conjugated with glucuronic acid in the liver and little is exerted unchanged in urine. Cirrhotices and neonates,who have low conjugating ability,require lower doses.The metabolite is exerted mainly in urine .Plasma $t_{1/2}$ of chloramphenicol is 3-5 hours in adults .It is increased only marginally in renal failure;dose need not be modified.

Absorption,fate and excretion

Chloramphenicol is completely absorbed from the gut and is better diffusible into the tissues. Blood levels of chloramphenicol after oral administration are superior to those after IM administration because of the hydrolysis of its salt in the gut and is better diffusible into the tissue. Being poorly water soluble, its absorption depends to a great extent on a particle size. the absorption, distribution, metabolism, and excretion of chloramphenicol. Chloramphenicol is rapidly absorbed from the gastrointestinal tract in humans and animals, with peak values in plasma being reached within two to three hours of administration. It is extensively distributed throughout the human body, regardless of its administration route, and has been found in the heart, lung, kidney, liver, spleen, pleural fluid, seminal fluid, ascitic fluid, and saliva. About 50% of chloramphenicol

in the blood is bound to albumin. Chloramphenicol penetrates the brain-blood barrier, and its concentration in cerebrospinal fluid can reach about 60% of that in plasma. The concentration attained in brain tissue equals or exceeds that in plasma. Chloramphenicol easily crosses the placenta and also is secreted in breast milk. Chloramphenicol has a half-life ranging from 1.6 to 4.6 hours (longer in neonates), with an apparent volume of distribution ranging from 0.2 to 3.1 L/kg. The half-life was longer following oral than following intravenous administration. Patients with chloramphenicol-induced bone marrow depression experienced reduced clearance rates. The primary metabolite of chloramphenicol is the glucuronide conjugate.

Chloramphenicol arylamide is formed by intestinal bacterial reduction of the nitro group of chloramphenicol to an amine, which is acetylated and excreted in the urine. Human liver microsomes can reduce the nitro group of chloramphenicol. Oxamic acid, oxamylethanolamine, and aldehyde derivatives also have been identified as metabolites of chloramphenicol. Chloramphenicol, its glucuronide conjugate, chloramphenicol base, and the oxamic acid, alcohol, acetylarylamine, and arylamine metabolites were found in the urine of rats administered ^{3}H -chloramphenicol intramuscularly. The major metabolites were assumed to be chloramphenicol base (~26%) and the acetylarylamine derivative (~20%) on the basis of recovered radioactivity. Similarly, chloramphenicol, its glucuronide conjugate, and the oxamic acid, acetylarylamine, arylamine, and base derivatives were found in the urine of goats administered chloramphenicol intramuscularly. Some of the chloramphenicol metabolites are more toxic than the parent compound and may be toxic to the bone marrow. For example, reactive nitroreduction intermediates have been associated with DNA damage. Dehydrochloramphenicol, a metabolite produced by intestinal bacteria, can undergo nitroreduction in the bone marrow. Individuals producing more of the toxic metabolites, or having a greater capacity for nitroreduction, could be

predisposed to stem-cell damage ultimately resulting in aplastic anemia and/or leukemia. **Identification of metabolites**

Chloramphenicol is eliminated primarily following biotransformation:

- In humans, as much as 90% of administered chloramphenicol is eliminated in urine as the chloramphenicol glucuronide conjugate.
- In other species (e.g., dog and rat), urinary elimination is dominant, but larger amounts are eliminated in bile as aromatic amines.
- In humans, as much as 10% of the administered dose may be eliminated unchanged in the bile.
- The direct conjugation to form glucuronide is at the primary rather than the secondary alcoholic group.

Use

Chloramphenicol is an antimicrobial agent with restricted use, because it causes blood Dyscrasia:

- It is used to combat serious infections where other antibiotics are either ineffective or contraindicated.
- It can be used against gram-positive cocci and bacilli and gram-negative aerobic and anaerobic bacteria. Chloramphenicol has been used since the 1950s to combat a wide range of microbial infections, including typhoid fever, meningitis, and certain infections of the central nervous system.
- It currently is used in eye ointments to treat superficial ocular infections involving the conjunctiva or cornea, in topical ointments to treat the external ear or skin, in various tablets for oral administration, and in intravenous (i.e.) suspensions to treat internal infections.
- Chloramphenicol also has been used in veterinary medicine as a highly effective and well-tolerated broad-spectrum antibiotic. Because of its tendency to cause blood dyscrasia in humans, its use in food-producing animals is now prohibited.
- Chloramphenicol still is used in cats, dogs, and horses to treat both systemic and local infections.
- Chloramphenicol also is used in ophthalmic preparations, including ointments, solutions, and drops. (Pediatric doses must be lower, to avoid gray baby syndrome. Gray baby syndrome is characterized by cardiovascular collapse in infants, apparently due to an accumulation of active, unconjugated chloramphenicol in the serum, resulting from its decreased glucuronide conjugation in the liver).

Analysis

Chloramphenicol can be detected in blood serum, plasma, or cerebrospinal fluid by high pressure liquid chromatography (HPLC). HPLC or enzyme immunoassay may be used to determine chloramphenicol levels in blood. Chloramphenicol can be measured in pharmaceutical preparations for humans and animals with microbiological, turbidimetric, and spectrophotometric assays. Thin-layer chromatography and densitometry are used in the analysis of prescription drugs. Chloramphenicol levels in meat, milk, and eggs have been determined with thin-layer HPLC and radioimmunoassay.

Environmental occurrence

Chloramphenicol may be released to the environment and may be found in various waste streams because of its use as a medicinal and research antimicrobial agent. Chloramphenicol also may be isolated from *Streptomyces venezuelae* in the soil.

Environmental fate

Chloramphenicol may be present in the environment because of releases into various waste streams. If released into the atmosphere, chloramphenicol will exist primarily in the particulate phase. Removal of atmospheric chloramphenicol would occur mainly through dry deposition. The atmospheric half-life of chloramphenicol is 12 hours, as it will react with photochemically produced hydroxyl radicals. If released to water, chloramphenicol will be essentially nonvolatile. Adsorption to sediment or bioconcentration in aquatic organisms are not expected to be important processes. If released to soil, chloramphenicol is expected to have high soil mobility. Volatilization of chloramphenicol is not expected from either dry or wet soils. Various biodegradation studies indicate that chloramphenicol may biodegrade in soil and water. Chloramphenicol degraded when adapted activated sludge was used as the inoculum. It also was degraded by intestinal bacteria via amidolysis; 18 metabolites were observed, with 2-amino-1-(*p*-nitrophenyl)-1,3-propanediol and its *p*-aminophenyl reduction byproduct as the major metabolites

Environmental exposure

Exposure to chloramphenicol may occur through inhalation, dermal contact, ingestion, or contact with contaminated water or soil. Because of potentially harmful effects to humans, chloramphenicol was banned by the Food and Drug Administration (FDA) in 1997 from use in food-producing animals (FDA 1997). No data on levels of chloramphenicol in food products were found in the literature.

Biological indices of exposure

Chloramphenicol can be detected in blood serum, plasma, cerebrospinal fluid, and urine. It is rapidly absorbed from the gastrointestinal tract and is distributed extensively through the human body, regardless of route of administration. It has been found in the heart, lung, kidney, liver, spleen, pleural fluid, seminal fluid, ascitic fluid, and saliva. Upon metabolism, chloramphenicol yields D-*threo*-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol and chloramphenicol-□-D-glucuronide. Around 90% of chloramphenicol is excreted in urine.

The majority of the release is in the form of metabolites, including conjugated derivatives, while only 15% is excreted as the parent compound. The half-life of chloramphenicol in adult humans ranges from 1.6 to 4.6 hours. Peak levels appear two to three hours after oral administration of chloramphenicol. In infants, chloramphenicol's half-life is much longer. The half-life ranged from 10 to > 48 hours in infants aged one to eight days and from five to 16 hours in infants aged 11 days to eight weeks. Regardless of its route of administration to humans, chloramphenicol is readily absorbed and extensively distributed. The major metabolite is the chloramphenicol glucuronide conjugate; however, several studies indicate that the dehydrochloramphenicol metabolite produced by intestinal bacteria may be responsible for DNA damage and carcinogenicity. This metabolite can undergo nitro reduction in the bone marrow, where it may cause DNA single-strand breaks and stem-cell damage leading to aplastic anemia and leukemia. Chloramphenicol inhibits mitochondrial protein synthesis, a process essential for normal hematopoiesis. Mitochondrial abnormalities induced by chloramphenicol are similar to those observed in preleukemia, suggesting that mitochondrial DNA is involved in the pathogenesis of secondary leukemia.

Material and method

Study subjects

In this experiment, *E. coli* cells were cultured under ideal growth conditions but in the presence of antibiotics as a selective environmental stress in order to select for resistance. *Escherichia coli* cells growing under ideal conditions are able to complete one reproduction cycle in as little as every twenty minutes. Since so many generations are able to be observed, one should theoretically be able to observe thousands of generations and determine evolution's effects over a short period of time.

Study plan

1. Preparation of culture of E.coli. using M-H media to obtain fresh colonies of it.
2. The *E. coli* was then transferred into M-H broth, and grown for 24 hours at 37˚C. This *E. coli* culture was used as the beginning stock culture for this experiment.
3. antibiotic sensitivity test is performed by using K-B technique on M-H media.
4. observation of inhibitory zone is done directly by measuring the diameter of inhibitory zone.
5. All steps were performed using aseptic technique.
6. continuously study the antibiotic sensitivity of E.coli. for chloramphenicol, by using E.coli. cell obtained form near to zone of inhibition.

Kirby-Bauer method

The Kirby-Bauer test, known as the disk-diffusion method, is the most widely used antibiotic susceptibility test in determine what treatment of antibiotics should be used when treating an infection. This method relies on the inhibition of bacterial growth measured under standard conditions. For this test, a culture medium, specifically the Mueller-Hinton agar, is uniformly and aseptically inoculated with the test organism and then filter paper discs, which are impregnated with a specific concentration of a particular antibiotic, is placed on the medium. The organism will grow on the agar plate while the antibiotic "works" to inhibit the growth. If the organism is susceptible to a specific antibiotic, there will be no growth around the disc containing the antibiotic. Thus, a "zone of inhibition" can be observed and measured to determine the susceptibility to an antibiotic for that particular organism. The measurement is compared to the criteria set by the National Committee for Clinical Laboratory Studies (NCCLS). Based on the criteria, the organism can be classified as being Resistant (R), Intermediate (I) or Susceptible (S).

Mueller-Hinton agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. Mueller Hinton Agar is often abbreviated as M-H Agar, and complies with requirements of the World Health Organization.

Chemical composition of M-H agar media

Beef Extract ..... 2 g
Acid Hydrolysate of Casein ..... 17.5 g
Starch ..... 1.5 g
Agar ..... 17 g
Final pH 7.3 ± 0.1 at 25°C

Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, and amino acids in Mueller Hinton Agar. Starch is added to absorb any toxic metabolites produced. Agar is the solidifying agent.
A suitable medium is essential for testing the susceptibility of microorganisms to sulfonamides and trimethoprim. Antagonism to sulfonamide activity is demonstrated by para-aminobenzoic acid (PABA) and its analogs. Reduced activity of trimethoprim, resulting in smaller growth inhibition zones and inner zonal growth,

is demonstrated on medium possessing high levels of thymide. The PABA and thymine/thymidine content of Mueller Hinton Agar are reduced to a minimum, reducing the inactivation of sulfonamides and trimethoprim.

Media preparation

Suspend 38 g of the medium in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. boil for 1 minute for complete dissolution. dispense into appropriate condition and sterilized by Autoclave at 121°C for 15 minutes. Cool to room temperature. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth. Allow to cool to room temperature. Check prepared Mueller Hinton Agar to ensure the final pH is 7.3 ±0.1 at 25°C. after preparation of media E. coli. are inoculated by spared plate method and wafers which are dipped on chloramphenicol are placed on the petriplates, now these Petri plates are left for incubation of 24 hrs. After incubation zone of inhibition is observed to determine antibiotic sensitivity of bacteria.

Results

Initial antibiotic sensitivity

The results for the antibiotic sensitivity test against chloramphenicol antibiotics is determine by measuring zone of inhibition in mm. for chloramphenicol antibiotic test on Mueller Hinton agar culture of *E.coli*. is observed approx. 23 mm. it shows *E. coli.* is sensitive for chloramphenicol. After 22 days, a slight decrease in the diameter of the zone of inhibition is observed, which is approx. 20-21 mm, and after 64 days the zone of inhibition decreases near to 17-18 mm, it shows decrease in sensitivity of *E.coli*. for chloramphenicol.

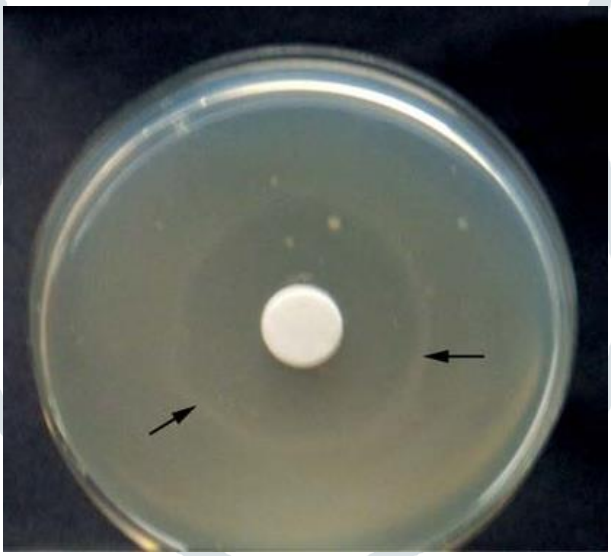


Figure shows zone of inhibition generated by chloramphenicol on Mueller Hinton agar media of E. coli.

Discussion

E. coli cultures were incubated for a total of 64 days. In this amount of time, *E. coli* showed a decrease in the diameter of the zone of inhibition and therefore a decrease in the sensitivity to chloramphenicol.

Our results show that *E. coli* has the capacity to have a decrease in antibiotic sensitivity over time, but whether or not it can become completely resistant to either antibiotic due to random mutations alone is inconclusive from our data.

However, the failure to observe a significant effect the overall study might be due to the relatively small time period of study. As this was only a pilot study done during the training period, therefore, additional studies with a greater number of subjects are needed to confirm these findings.

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