To Study the Toxic Effect of Metal Ion Chelators on *Pasteurella multocoda*

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Summary: The aim of our research was to check the effect of metal ion chelators on the growth of *Pasteurella Multocida*, either they can enhance or inhibit the growth. The organism grow more rapidly when they were treated with the metal ion chelators. The chelators used were salicylhydroxamic acid,oxine and EDTA.

Introduction

Pasteurella multocida (Pm) is a short encapsulated gram-negative rod that exhibits the bi-polar staining. It is a normal flora genus in the mouth of many animals especially domestic animals particularly cats and dogs and is transmitted by biting, about 25 % of animal bites becomes infected with the organism. Most of the bite infections are polymicrobial with a variety of facultative anaerobes and aerobic organisms present in addition to Pasteurella multocida Pathogenesis is not well understood except the capsule is a virulent factor and endo- toxin is present in the cell wall. No exo-toxins are made. Pasteurella multocida is virulent to many species of birds & animals causing hemorrhagic septicemia [1]. *Pasteurella multocida* may present normally cattle, sheep, swine, dogs and cats. It is present in the teeth and saliva of these animals. It causes respiratory tract & nasal sinus of persons associated with animals, causing pneumonia bronchitis & nasal sinusitis [2]. Cellulitus is the disease caused by bite of animals to the human. The rapid on set is indicative of Pm infection. Cat's sharp pointed teeth can implant the organism under the skin [3]. There is no vaccine against *Pasteurella multocida* infection but ampicillin is given to the patient to prevent the infection. Sulfonamides, tetracyclin & streptomycin can be given because they are also sensitive to *Pasteurella multocida* in vitro [4]. Metal ions are involved in all aspects of life. The alkali metals, alkaline earth metals and a number of transition elements are all essential metals, although they are present only at ultra trace level. These cations have variety of functions [5,6]. Metal ion chelators themselves may cause growth inhibition, which can be relieved by addition of further metals e.g. the growth of Legionella pneumophilia in a complete defined medium is particularly prone to inhibit by EDTA or citrate, inhibition can be reversed by adding compounds of cobalt, copper, iron or other metals [7]. In iron deficient envoirment microorga-nism produces a low molecular weight chelating agents called sidero-hores or microbial iron chelators to solublise and transport ferric ions in aqueous medium [8]. The overload of iron in living organism some times becomes a serious problem. Using siderophores or iron chelators may solve the problem. Enterobactin Ferrioxamine and salicylichydroxamate are the examples [9]. The Toxcity of metal compounds has important implication in human and animal health, agriculture ecological processes and biotechnology. Sidero-hores are best chelators for iron but they also disturb the equilibrium of other metal ions in living system. They also form complexes with other trace metals present in the body. Co-ordination of metal ions by siderophores may be desirable in view of the potential clinical significance. When a host is challenged by siderophores either as a drug for iron over dose or as a result of microbial invasion, balance metals may be disturbed [10]. Stability constants or equilibrium constant for metal complex formation have long been employed as an effective measure of the affinity of a ligand for a metal ion in solution [11]. The aim of our research was to check the effect of metal ion chelators on the growth of *Pasteurella Multocida*, either they can enhance or inhibit the growth of the organism more rapidly when they were treated with metal ion chelators. The metal ion chelator used were salicylhydroxamic acid, oxine and EDTA.

Results and Discussion

It was quite obvious by observing the readings of absorbance with EDTA, salicylhydroxamate and oxine, these metal ion chelators inhibited or controlled the growth of Pasteurella multocida.Table-1 and Fig.1 showed the effect of EDTA on

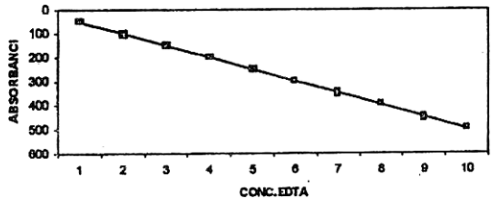

Fig. 1: Growth of PM. Diff. Conc. EDTA.

P.multisoda. When the experiments were carried out with EDTA , the first reading in Table No.(1) showed the maximum growth of *Pasteurella multocida*, the concentration of EDTA in 2nd tube was minimum that was only 50 µl while the last tube that was tube no 11th showed the minimum growth of *Pasteurella multocida*, this tube contained the maximum concentration of the EDTA, that was 500 µl. From test tube no 3 to 7 it was a stationary phase as obvious from the readings, from tube no 7 to 11 the growth is inhibited or it can be considered as a death phase. Similar results showed when the procedure was carried out with salicylhydroxamate. This metal ion chelator also proved to inhibit the growth of Pasteurella multocida at maximum level. Table-2 and Fig. 2 showed the effect of salicylhydroxamate on the growth of *P.mutocida.* The 1st reading in the Table No. 2 showed the sufficient growth of *Pasteurella multocida,* which was with out the metal ion cheleator. Reading 2nd to 5th showed the stationary phase, which means although the microbes are metabolically active but not in a position to carry out growth or rate of growth is extremely low. The Table 3 and Fig. 3 show the effect of oxine on the growth of *P.multocida.*

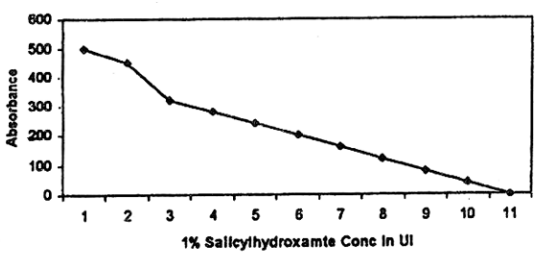

Fig. 2: Ab sorbance with Salicyl Hydroxamate.

Readings 2nd to 11th showed clearly that the growth of microbes is totally inhibited. In tube no 11, the growth of *Pasteurella multocida* is almost nil as it contained the highest concentration of oxine that is

Table –1: Absorbance of Growth with EDTA .

Tube No.	BHI media taken	1 % EDTA Conc. µl	Absorbance at 520 nm .
1.	10 ml	control	0.102
2.	10 ml	50	0.81
3.	10 ml	100	0.79
4.	10 ml	150	0.78
5.	10 ml	200	0.76
6.	10 ml	250	0.74
7.	10 ml	300	0.71
8.	10 ml	350	0.67
9.	10 ml	400	0.65
10.	10 ml	450	0.63
11.	10 ml	500	0.60

Table-2: Absorbance Growth with Salicylhydroxamate

Tube No.	BHI media Taken in ml	1 % Salicyl -hydroxamate Conc. In µl	Absorbance at 520 nm
1.	10 ml	control	0.110
2.	10 ml	40	0.90
3.	10 ml	80	0.88
4.	10 ml	120	0.85
5.	10 ml	160	0.84
6.	10 ml	200	0.82
7.	10 ml	240	0.72
8.	10 ml	280	0.69
9.	10 ml	320	0.64
10.	10 ml	450	0.59
11.	10ml	500	0.56

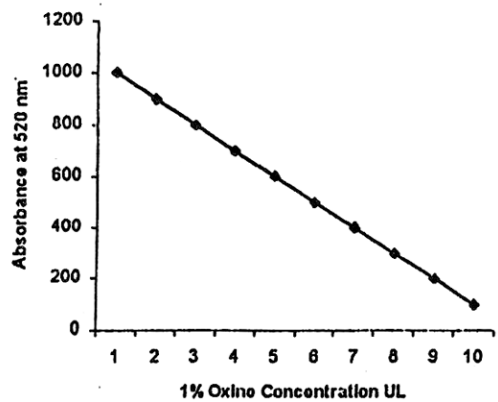

Fig. 3: Absorbance of Growth with Oxine.

400µl. So we can consider from tube no 6th to 11th a 'death phase'. *Pasteurella multocida* behaved in a similar pattern with 3rd metal ion cheleator that was oxine. Although oxine was found not to be very efficient in inhibiting or controlling the growth of Pasteurella multocida, it showed the positive results. Figure 4 showed the comparative study of anti microbial activity or metal ion chelator, among these

Table-3:Absorbance of Growth with Oxine.

Tube No.	BHI media Taken.	1 % Oxine Conc.µl	Absorbance at 520 nm
1.	10 ml	control	0.103
2.	10 ml	100	0.95
3.	10 ml	200	0.94
4.	10 ml	300	0.92
5.	10 ml	400	0.90
6.	10 ml	500	0.89
7.	10 ml	600	0.87
8.	10 ml	700	0.85
9.	10 ml	800	0.81
10.	10 ml	900	0.77
11.	10ml	1000	0.76

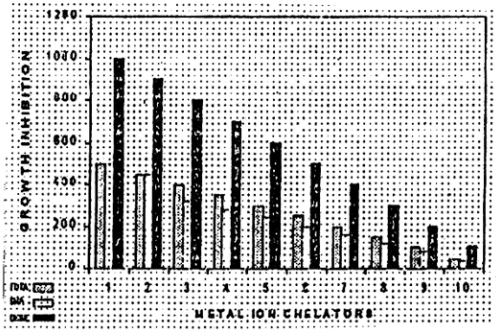

Fig. 4: Antibecterial Activity of Metal Ion Chelaors on PM.

three compounds oxine showed very low while salicylhydroxamate had maximum growth inhibiting activity. EDTA had very close to salicylhydroxamate.

The above research work concluded that metal ion chelators can act as anti bacterial agents in vitro. EDTA and salicylhydroxamate were found to be very harmful in inhibiting the growth of *Pasteurella multocida* and can be used as bacteriocidal. Oxine is also considered to be effective but not so efficient. Hence it can be hypothesized from the experiments being performed that that metal ion chelators namely, EDTA, salicylhydroxamate and oxine to some extent can be used against the control of *Pasteurella multocida*'s infection. Various vaccines can also be prepared on the laboratory level as a protectory measures from the diseases caused & spread by *Pasteurella multocida.*

Experimental

Materials

(1). BHI Broth (g / l)

a. Calf Brain Infusion solid = 12.5 g (Oxoid)

b. Brain Heart Infusion solid = 5.0 g (Oxoid)

c. Protease peptone = 10.0 g (Oxoid)

d. Glucose = 2.0 g (Oxoid)

e. NaCl = 5.0 g (Oxoid)

f. Di-sodium phosphate = 2.5 g (Oxoid)

(2). CYS Media (g / 300 ml)

a. Casein hydrolysate = 0.9 g (Gibco)

b. Sucrose = 1.8 g (Merck)

c. Yeast extract = 1.8 g (Merck)

d. NaCl = 1.5 g (Merck)

e. Di pot. hyd. phosphate = 2.58 g (Merck)

f. Pot. hydrogen phosphate = 39 g (Merck)

(3) Ethylene diamine tetraacetic acid (EDTA)

(4) Salicylhydroxamate

(5) Oxine

(6) Crystal violet

(7) Ethyl alcohol

(8) Ammonium oxalate

(9) Aqua dust

(10) Iodine

(11) Potassium iodide

(12) Acetone

(13) Safranine

Growth of Pasteurella multocida

In two conical flasks of 500 ml, 300 ml of distilled water was taken and both the media's , CYS & BHI broth were dissolved. After preparation of media flasks were tightly closed and covered with cotton and aluminum foil and put in to a autoclave

for sterilization at 180°C for one hour. After cooling the flasks some of the cells of Pasteurella multocida were inoculated in the flasks under the laminar and kept the flasks in incubator for 48 hours at 37°C. After 48 hours both the medias were checked by gram staining, pink colored bi-polar non motile mass confirmed the growth of Pasteurella multocida. Physically the color of medias were also changed which turned in gelatinous white from brown [12].

Gram Staining :

Principal Stain

Sol (1): Crystal violet 10gm

Ethyl alcohol 100ml (95 %)

Sol (2) : Ammonium oxalate 1gm

Aqua dust 100ml

Mix 20 ml of sol (1) with 80 ml of sol (2) to make principal stain .

Mordant :

Sol (3) : Iodine 1g

Pot . Iodide 2g

Grind and mix iodine and potassium iodide thoroughly in a grinder , then dissolve in 200 ml aqua dust .

Decolorizer

Sol (4) : *Ethyl alcohol.....250ml (59 %)*

Acetone..... 250ml

Counter Stain

Sol (5) : Safranine.....2.5g

Ethyl alcohol.....100ml

Dilute sol (5) on raio of 1:4 with aqua dus [13].

Staining Procedure

1. Heat fixation of the slide (move three time through flame).
2. Cover the slide for 30 seconds with mixing of principal stain.
3. After 30 seconds rinse with solution 3 .
4. Let the slide be covered for 60 seconds with sol. 3.
5. After 60 seconds rinse the slide with distilled water .
6. Decolorize by treating once or twice in sol. 4 and immediately taken out .
7. Immediately after decolorizing cover slide with sol 5 for 30 seconds .
8. After 30 seconds, rinse with distilled water dry carefully filter paper [13] .

The cultured media were centrifuged at 3000 rpm for 20 min to collect the cells. After collection of the cell lyophilization of the sample was done.

Growth with Metal Ion Cheleators (Edta Oxine, Salicylhydroxamic Acid)

1. 10 ml of BHI broth in 11 tubes .
2. Addition of 1 % EDTA or oxine or salicyl-hydroxamic acid solution (1g + 99 ml distilled water) with the increment of 50 µl in 10 tubes, while the 1st is control (with out metal ion cheleators solution).
3. Autoclaved for 1 hour at 180°C .
4. Inoculation of the cells of the *Pasteurella multocida.*
5. Incubated for 48 hours at 37°C .
6. Checked the growth by two methods.

(A). Gram staining

(B). Spectrophotometric technique (absorbance at 520 nm) gram staining confirmed the bi-polar growth of *Pasteurella multocida*

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