Inhibition of Growth of *Streptococuss pyogenes* Group A and *Escherichia coli* by EDDA

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Summary: The growth inhibiting activity of EDDA (Ethylenediamin di-(*o*-hydroxyphenyl-acetic acid) towards *Streptococuss pyogenes* group A and *E. coli* MW was investigated. Bactericidal effect was observed at concentration up to 14 mM in *E. coli* MW whereas the strains of *Streptococcus pyogenes* group A were unaffected by 44 mM EDDA suggesting that *Streptococcus pyogenes* group A was insensitive to even large molar excess of EDDA and this observation indicated either extraordinary efficient Fe acquisition or low Fe requirement as compared to *E. coli* MW.

Introduction

Iron is an absolute requirement for the growth of most microorganisms, with the possible exceptions of lactobacilli [1] and *Borrelia burgdorferi* [2]. Iron is considered as an important ingredient in the growth of various microorganisms as it is essentially required for some bacterial enzymes [3]. Under aqueous, aerobic conditions and at neutral pH, the concentration of free iron in the human body is estimated to be 10^{-18} M, a concentration that is several orders lower than that required to support a productive bacterial infection. To overcome the problem of iron scarcity, pathogenic bacteria have evolved efficient strategies for obtaining iron from human and animal hosts by extracting the metal from eukaryotic proteins, such as transferrin and lactoferrin. To extract iron from these proteins, bacteria secrete small organic chelators called siderophores which can bind iron with association constants as high as $\sim 10^{50}$ [4]. To understand the effect of iron on the growth of microbes *in vitro*, normally test microbe is exposed to either iron-deficient or iron depleted or iron-replete medium. For this purpose, the different chelators are incorporated in media to assess the growth kinetics of bacteria. It is generally accepted that in iron-deficient medium, the siderophores are excreted from the bacteria in order to make complex with surrounding iron and the same complex (siderophores + iron) is retransported to bacterial cells. For this purpose, it is essential to know the concentration of chelators which enable medium iron –deficient or iron-depleted.

Streptococuss pyogenes Group A is major etiological agent causing a variety of human diseases ranging from Pharyngitis to severe and life threatening invasive disease, such as toxic shock-like syndrome (TSLS) and necrotizing fasciitis [5].

The objective of present study was to investigate the effect of different concentrations of EDDA (Ethylenediamin di-(*o*-hydroxyphenylacetic acid), iron chelator, on the growth of both *Streptococuss pyogenes group* A and *Escherichia coli* MW by using both growth kinetics and well plate technique.

Results and Discussion

Repeated experiments (three times) with any of streptococcal isolates shown in (Table-1) failed to detect any inhibition of bacterial growth under conditions of iron-restriction. From results presented in (Fig.1), it was concluded that 27 strains of *S. pyogenes* GrA were able to grow unrestrained under iron-limited conditions, with a 10 to 20 molar-excess of EDDA over Fe as well as in iron replete media. The growth of *E. coli* MW was inhibited at a 10 fold molar excess of the chelator (Fig. 2). The growth by EDDA in the agar plate assay was performed on various strains of *Streptococuss pyogenes* Group A, and *E. coli* MW. Fig. 3 showed the zone of growth inhibition of *E. coli* MW after 18 h at 37[0] C in the presence of various concentrations of EDDA. All
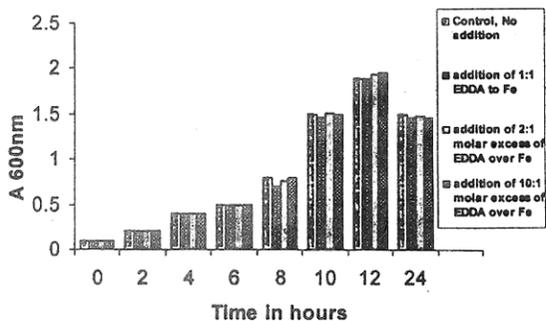
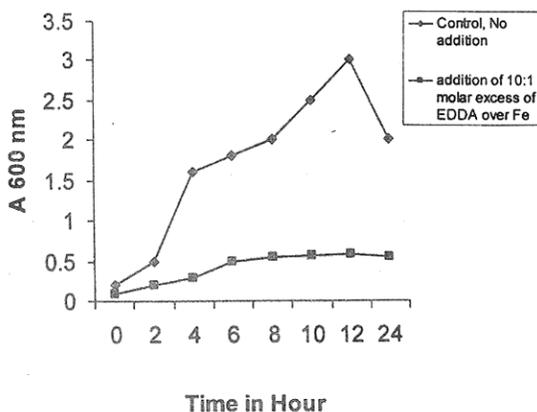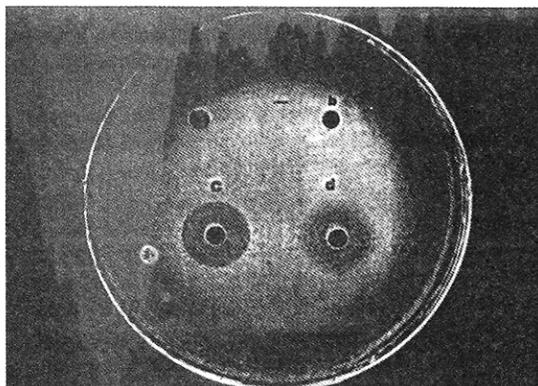Fig: 1:   Growth kinetics of *S. pyogenes* strain 55903Min B. H. I. Broth.

Fig: 2:   Growth kinetics of *E. coli* in B. H. I. Broth.

Key. Effect of EDDA on the growth of *E.coli* MW
a=5 µl of 1.5 mM EDDA, b=5µl of 4.4 mM EDDA, c=5 µl of 14 mM EDDA, d=10 µl of 14 mM EDDA

Fig. 3:   Plate assay for inhibition of growth by EDDA.

strains of *S. pyogenes* were unaffected by concentrations of EDDA by 14 mM EDDA. Growth of all strains of *S. pyogenes* was unaffected by concentrations of EDDA up to 44 mM. An example of the pattern of the growth on the plate assay for strain 55903M is shown in Fig.4. These observations correspond with the findings of Marcelis *et al.* [6], where *S. faecalis* was shown to be insensitive to EDDA up to 44 mM.

It is generally accepted that EDDA, strong iron chelator, can inhibit/limit the growth of many Gram-positive and Gram negative bacteria *in vitro*. Under such iron limiting conditions bacteria produce siderophores for acquiring iron for their growth. Previous workers [7-8] demonstrated that EDDA could limit/inhibit the growth of many bacteria by binding the iron available in the medium.
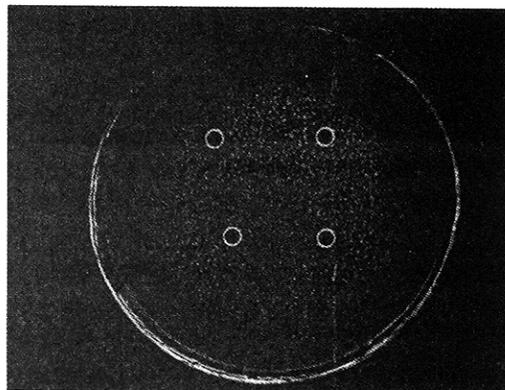
Key. Effect of EDDA on the growth of *Strep.pyogenes* GrA
a=5 µl of 1.5 mM EDDA, b=5µl of 4.4 mM EDDA, c=5 µl of 14 mM EDDA, d=10 µl of 44 mM EDDA

Fig. 4:   Plate assay for inhibition of growth by EDDA.

The findings presented in this paper of *Streptococcus pyogenes* Group A are not surprising as the strains of *Lactobacillus*, *Pedicoccus*, *Leuconostoc*, were able to compete and grow in the presence of chelators [9] and none of the strains of *S.pyogenes* tested in this study produced sidero-phores. Our findings coincide with the observations of Neilands [10]. Evans *et al.*, [11] and Francis *et al.*, [12] that *S. mutants* and *S. pyogenes* did not produce ferric chelating and transporting sidero-phores respectively. These data confirmed that a high affinity siderophore-mediated iron acquisition system was not involved. A large molar excess of EDDA in

plate technique had no significant effect (appearance of zones) indicate either extraordinary efficient Fe-acquisition or a very low or zero Fe requirement. Iron requirement and production of siderophores in *E. coli* [13] and *Staph. aureus* [14] are well documented.

Metal ions such as manganese, copper, iron, cobalt, and zinc are essential trace elements but are also potentially harmful, which necessitates careful regulation of metal homeostasis [15]. Several species of streptococci can grow in the absence of iron [16, 17], and it has been proposed that Mn can replace iron [18]. A connection between Mn homeostasis and sensitivity to oxidative stress has been reported [19, 20]. *S. pyogenes* lacks catalase but produces a Mn-dependent SOD [21, 22]. In contrast to many other species which have several SODs, with different cofactors (Mn, Fe, and Cu/Zn), it has been suggested that *Bacillus subtilis* and most streptococci and enterococci mainly utilize the Mn SOD [17, 23].

Although considerable work has been done on iron and its acquisition by invading bacteria, little is known about the requirement and acquisition of other metals by bacteria. It is well accepted that iron plays an essential role in some microbial enzymes, yet insufficient data is available on the role of other metals. The findings of this study regarding *S. pyogenes* draws attention to study the role of other metals in microbes where iron is either replaced by other metals or is not absolute requirement.

Experimental

All glassware was immersed in 1 M HCl for 24 h and rinsed thoroughly six times with deionized water before being sterilized by dry heat at 160 ^{0}C for 2 h before use. All solutions were made in glass distilled and deionized water.

Bacteria

Before use Streptococcal GrA strains and *E.coli* MW (Table 1) were cultured in either Brain Heart Infusion Broth (BHI, from Oxoid), or Todd Hewit Broth (THB, from Gibco) for 12 h at 37^0 C. After 12 h of incubation, bacteria were harvested by centrifugation at 5000 rpm washed twice with sterile saline which was used as inoculum.

Assay

Table-1: Bacterial strains-Designation and origin

Hospital No.	Organism	Site	Source
S. Pyogenes	Gr A		
54359V	"	Not known	GRI
55903M	"	.	"
60343X	"	"	"
88/00657	"	"	RH
88/00657	"	"	"
52114	"	"	CL
45713/87	"	"	"
52011	"	Throat swab	"
45543/87	"	Not known	"
47061	"	Swab from scalp	"
48749	"	Ear swab	"
48137	"	"	"
5790	"	"	"
11324	"	Wound swab	"
13151	"	Throat swab	"
9242	"	Discharge from sore	"
12757	"	Swab from vulva	"
12997	"	Throat swab	"
10132	"	"	"
5790	"	Pus	"
52986	"	Nail infection	"
53141	"	Pus from skin wound	"
52942	"	Otitis media	"
1537	"	Otitis media	"
1594	"	Not known	"
2750	"	"	"
02750	"	"	"
E. coli MW		"	Not known

NK	Not known
GRI	Department of Bacteriology, Royal Infirmary, Glasgow
	Department Laboratory Medicine, Ruchill Hospital
RH	Glasgow
	Bacteriology South Lannarkshire Laboratory, Law
CL	Hospital, Carluke.

EDDA (Sterilized by membrane filtration 0.4 µm pore size Millipore) was added to each medium at concentrations of equimolar, or 5, 10, and 20 fold molar excess of Fe for the growth kinetics purpose. The broths were stored at 4^{0}C for 24 h in order to allow further binding of Fe in the medium. Growth was estimated by measuring A_{600nm} of samples withdrawn at 1h or 2h intervals; when A_{600nm} exceeded 2, the culture was diluted with appropriate sterile medium until the absorbance value was brought into the range of 1. Absorption values were measured on an SP6-550UV/VIS spectrophotometer (Pye Unicam). The appropriate uninoculated medium served as a blank and the growth curves were plotted graphically.

medium was changed to nutrient agar. Nutrient agar (15 ml) was poured into plastic petridishes (6.8 cm diameter), resulting in an agar layer of 5 mm thickness. Wells of 3 mm were cut out of the agar and the plates were flooded with 3 ml of 10^{-2} dilution of an 18 hour culture of bacteria in nutrient broth. After removal of the excess fluid, especially from the cups, the surface of the agar plates contained approximate 10^{-2} bacteria, sufficient to produce confluent growth. After drying the plates, (1 h at 45^0C), 5 µl of solutions containing EDDA ((Ethylenediamin di-(o-hydroxyphenylacetic acid, Sigma), 1.5, 4.4, 15, and 44 mM) were pipetted into the wells. Zones of growth inhibition in the lawn were observed after 10-18 h at 37^0C.

Acknowledgement

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