August 2010

Ph.D. Dissertation

Evaluation of chemotherapeutic combinations against *Vibrio vulnificus* infection

Graduate School of Chosun University

Department of Medicine

Ganesh Prasad Neupane

Evaluation of chemotherapeutic combinations against *Vibrio vulnificus* infection

August 25 2010

Graduate School of Chosun University

Department of Medicine

Ganesh Prasad Neupane

Evaluation of chemotherapeutic combinations against *Vibrio vulnificus* infection

Advisor: Professor, Dong-Min Kim, M.D., Ph.D.

April 2010

Graduate School of Chosun University
Department of Medicine
Ganesh Prasad Neupane

This is to certify that the Ph.D. dissertation of Ganesh Prasad Neupane has successfully met the dissertation requirements of Chosun University.

Committee chairperson   ·····························
                        (Prof., Cheol-Hee Choi, M.D., Ph.D.)
                            Department of Medicine

Committee member   ·····························
                        (Prof., Sook-Jin Jang, M.D., Ph.D.)
                            Department of Medicine

Committee member   ·····························
                        (Prof., Sung-Chul Lim, M.D., Ph.D.)
                            Department of Medicine

Committee member   ·····························
                        (Prof., Sung-Heui Shin, M.D., Ph.D.)
                            Department of Medicine

Committee member   ·····························
                        (Prof., Dong-Min Kim, M.D., Ph.D.)
                            Department of Medicine

June 2010

Graduate School of Chosun University

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C	Degree Celcius
μM	Micro mole
μL	Micro liter
μg/mL	Microgram per milliliter
ATCC	American Type Culture Collection
KCTC	Korean Collection for Type Cultures
$C_7H_{15}NO_4S$	Morpholinepropanesulfonic acid
$C_6H_{13}NO_5$	Tricin
CCl_4	Carbon tetra chloride
CDC	Center for Diseases Control and Prevention Centre
CFU/mL	Colony forming Units/ milliliter
CLSI	Clinical and Laboratory Standards Institute
CPS	Capsular lipopolysaccharide
CUH	Chosun University Hospital
CIP	Ciprofloxacin
CTR	Ceftriaxone
α, α' DD	Alpha, alpha dipyridyl
DFO	Deferoxamine
DFP	Deferiprone
DFS	Deferasirox
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dTTP	Thymidine triphosphate
dUTP	Deoxyuridine triphosphate
DX	Doxycycline
EMA	Ethidium bromide monoazide
$FeCl_3$	Iron chloride/Ferric Chloride
hr	hour
ICR	Imprinting Controlled Region
IPLM	Iron Poor Liquid Medium
i.m.	Intramascular
i.p.	Intraperitoneal
i.v.	Intravenous

K_2HPO_4	Potassium hydrogen phosphate
LPS	Lipopolysaccharide
LD_{50}	Lethal Dose 50
LB	Luria Bertani
mM	Millimole
mL	Milliliter
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MHB	Mueller-Hinton broth
NaCl	Sodium chloride
$Na_2S_2O_3$	Sodium thio sulfate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NTBI	Non-transferin-bound iron
ppb	Parts Per Billion
p.o.	Per Os
qPCR	Quantitative real time PCR
RTX toxin	repeats in toxin
SD	Standard Deviation
SPF/VAF	Specific pathogen free/Viral antibody free
TBI	Transferrin Bound Iron
TG	Tigecycline
TSA	Tryptic Soya Agar
TNF-alpha	Tumor Nnecrosis Factor alpha
TSS	Toxic Shock Syndrome
V. vulnificus	*Vibrio vulnificus*

초록

비브리오 패혈증의 효과적인 치료약제에 대한 평가

거네스 포러사더 네오파네
지도교수 김동민, M.D., Ph.D.
의과대학
조선대학교 대학원

Vibrio vumlnifucus 에 의한 패혈증은 만성 간질환자나 면역저하자에서 해산물을 날로 섭취했을 때나 *V. vulnificus*가 포함되어 있는 해수에 노출 되었을 때 발생한다. 간질환을 가지고 있는 환자의 경우 *V. vulnificus* 패혈증의 사망률은 50%이상이므로 사망률 감소에 있어서 신속한 진단과 적절한 항생제투여가 필수적이다.

*V. vulnificus*는 증식에 철이 필요한 균이고, 패혈증 환자의 약75% 에서 혈청 철의 상승이 확인되었다. 세 가지 iron chelating 약인 deferoxamine, deferiprone, 과 deferasirox을 가지고 *In vitro* iron chelation 실험으로 행하였다. 경구 철 착화제인 deferiprone 과 deferasirox는 *V. vulnificus*의 성장을 감소시켰으나, deferoxamine 은 오히려 *V. vulnificus* 의 성장을 촉진시켰다. 과잉철 상태의 질환에서 철 착화제로 dereroxaime 보다는 deferiprone 과 deferasirox 의 사용이 적절하리라 사료된다.

병합요법은 급속도로 치명적인 질환의 사망률을 감소시키기 위해 자주 사용된다. Ciprofloxacin(CIP)와 deferasirox(DFS)를 혼합한 In vitro time-kill 연구에서 24시간에 박테리아 수를 $2\log_{10}$ CFU/mL 이상으로 감소하여 *V. vulnificus*에 걸리기 쉬운 환자들의 치료에서, 항균 요법의 새로운 접근을 제시함으로써 새로운

형식의 synergism을 보여주었다. 또한 *V. vulnificus*에 대한 ciprofloxacin(CIP)과 tigecycline(TG)이 포함된 in vitro time-kill 실험에서는 24시간에 박테리아 수를 $3\log_{10}$ CFU/mL 이상으로 감소시킴으로써 synergism을 보였다.

In vivo 실험에서 철이 투여된 ICR 쥐들은 *V. vulnificus* 감염이 쉽게 되었고, LD_{50}은 10^1 에서 10^0 CFUs까지 감소되었다. Kaplan-Meier 생존 곡선에서 CIP경우 48시간에서(p=0.0002) 30%의 높은 생존 비율로 단독요법에서 가장 효과적인 약임을 보여주었다. 그러나 병합요법에서 doxycycline(DX IP) plus ceftriaxone(CTR)의 경우 마우스의 생존율이 50%(P=<0.0001)를 보였다. CTR plus DX PO와 CIP plus CTR은 mice생존에 효과적이지 않았다. 균 접종후 12시간째 혈액을 이용한 qPCR 검사에서 죽은 마우스의 혈액내 DNA copies 수는 최소 10^4 copies/uL 이었으나, 생존한 마우스의 DNA copies 수는 $10^2 \sim 10^3$ copies/uL을 보였다. 그러므로 혈액 내에 존재하는 DNA는 mouse에서 질병의 심한 증세를 예견하는 지표로 이용가능하리라 사료된다.

결론적으로 수혈을 받고 있거나 지중해 빈혈을 가지고 있는 환자처럼 *V. vulnificus*에 걸리기 쉬운 혈중 철농도가 높은 환자의 경우 DFS or DFP를 이용하여 착화요법을 하는것이 바람직하며, DX IV이 없는 경우, *V. vulnificus* 감염의 사망률을 줄이기 위해서는 DX PO보다 CIP을 투여하는것이 생존률 향상에 도움이 되리라 사료된다.

ABSTRACT

Evaluation of chemotherapeutic combinations against *Vibrio vulnificus* infection

Ganesh Prasad Neupane

Advisor: Prof. Dong-Min Kim, M.D., Ph.D.

Department of Medicine,

Graduate School of Chosun University

Background. *Vibrio vulnificus* is an emerging opportunistic human pathogen that can be life-threatening, particularly in patients with chronic diseases or compromised immune systems. *V. vulnificus* infection generally occurs following consumption of contaminated, raw seafood or through open wounds immersed in waters containing a high density of *V. vulnificus*. The mortality rate is greater than 50% in patients with liver diseases, and it may be further increased (67%) in patients who develop septicemia. *V. vulnificus* is ferrophilic organism, and almost 75% of infections progressing to septicemia are associated with iron overload. Hence, rapid diagnosis and administration of appropriate antibiotics are essential for reducing the mortality associated with this pathogen.

Methods. *In vitro* iron chelation experiments were performed to compare the efficacy of three different iron chelating drugs, deferiprone (DFP), deferasirox (DFS), and deferoxamine (DFO), to inhibit growth and reduce virulence of *V. vulnificus* organisms. *In vitro* time-kill studies were compared using either the bactericidal antibiotic ciprofloxacin (CIP) in combination with DFS or a combination of CIP and tigecycline (TG), a new glycylcycline class of antibiotic. *In vivo* mouse studies were performed to determine the efficacy of highly used antibiotics against *V. vulnificus* as assessed with a Kaplan-

Meier survival curve. Following infection, qPCR was performed to measure the bacterial loads in surviving and nonsurviving mice.

Results. *In vitro* iron chelation experiments demonstrated that the oral drugs DFP and DFS reduced the growth of *V. vulnificus*, which suggests that these drugs might be effective inhibitors of ferrophilic pathogens. *In vitro* time-kill studies using a combination of CIP and DFS or CIP and TG exhibited synergistic effects, reducing bacterial counts by $\geq 2\log_{10}$ CFU/mL at 24 hr. The synergistic effects of CIP combined with DFS were novel and suggest a new antimicrobial treatment for *V. vulnificus* infected iron overloaded patients. The Kaplan-Meier survival curve suggested that, CIP was an effective monotherapeutic drug associated with a survival rate of 30% at 48 hr ($P = 0.0002$). However, a single dose of intraperitoneal doxycycline (DX) combined with ceftriaxone (CTR) improved the survival of mice by 50 ($P = <0.0001$). Neither CTR combined with oral DX nor CIP combined with CTR were effective in terms of mouse survival. As measured by qPCR, the number of DNA copies in blood of single-dose antibiotic-treated nonsurviving mice at 12 hr were $\geq 10^4$ copies/μL in contrast to 10^2 □ 10^3 copies/μL in survival mice.

Conclusions. DFS or DFP are more effective therapies for *V. vulnificus*-susceptible, iron-overloaded patients receiving regular blood transfusions or with thalassemia. In unavailable condition of intravenous DX, CIP alone might be superior to DX p.o. for lowering the mortality of *V. vulnificus* infection. Furthermore, qPCR can accurately quantify changes in circulating bacterial counts and help clinicians predict disease severity and guide an aggressive treatment plan, if needed.

1.   INTRODUCTION

1.1   Background

V. vulnificus, an emerging opportunistic human pathogen can be life-threatening to immunocompromised patients, and especially in patients with liver cirrhosis, cancer, hepatitis B or C, adrenal insufficiency, hemochromatosis or other blood disorders, alcoholism, HIV/AIDS, gastric disorders, chronic renal diseases, malignancy, or diabetes [1-4]. *V. vulnificus* infection occurs following the consumption of raw seafood, particularly oysters, or from exposure of open wounds to waters containing a high density of *V. vulnificus* [5]. Clinically, the infection is characterized as severe, invasive, and life-threatening, in contrast to infections with other *Vibrio* species [6]. This virulent, halophilic, Gram-negative bacterium produces three perceivable syndromes [7]: (1) primary septicemia following consumption of raw or undercooked seafood; (2) wound infection resulting in cellulitis after an open wound is exposed to warm seawater highly concentrated with *V. vulnificus*; and (3) gastrointestinal illness characterized by vomiting, diarrhea, or abdominal pain. *V. vulnificus* infection is the leading cause of death related to seafood consumption in the United States [3]. Almost 67% of *V. vulnificus* infections are fatal owing to rapid and extensive tissue damage. The prognosis is directly linked to the accuracy of diagnosis and aggressive treatment. The mortality rate in shellfish consumption cases is approximately 53%, but it is even higher (67%) in patients with liver disease in whom *V. vulnificus* septicemia develops [8]. The mortality rates may increase to 100% in patients with septicemia if treatment is delayed by 72 hr [9]. Starks *et al.* [5] reported that death can occur within 24 hr after exposure to *V. vulnificus*. Infected individuals typically exhibit fever, chills,

hypotension, and characteristic bullous skin lesions, which are rapidly progressive with major damage and pathology.

The first description of *V. vulnificus* etiology dates back to 5th century B.C. when Hippocrates mentioned a man with a "violent pain in his foot" who also experienced symptoms of shivering, nausea, and fever followed by a disturbance in consciousness [10]. The patient died the following day when his foot became erythematous and swollen with black blisters. In 1970, Roland [11] examined a patient with a generalized papular hemorrhagic rash, vomiting, diarrhea, and fever who subsequently developed gangrene in the left leg and endotoxic shock. These symptoms were most likely caused by *V. vulnificus* infection. Initially the bacterium was identified as a "halophilic, lactose-positive marine *Vibrio*" and was named *V. vulnificus* in only 1979 by Farmer [12].

In 1976, Hollis and coworkers [13] of The Centers for Disease Control and Prevention (CDC) first characterized *V. vulnificus* as a lactose-positive bacterium, and the clinical syndromes associated with the disease were described by Blake *et al* [14]. In 1978, Matsuo *et al* [15] reported the first case of *V. vulnificus* in Japan. The first published report of *Vibrio* infection in Korea dates back to 1982 [16]. *Vibrio* infection is more prevalent in Korea than Japan and United States because of widespread consumption of raw seafood, even in inlands, and owing to a high prevalence of underlying liver diseae [17]. Yuan *et al* [18] in 1985 first reported *V. vulnificus* disease in Taiwan and is prevalent as in Korea. Infections of *V. vulnificus* now have been documented worldwide with an increased number of cases over the past 2 decades which may be attributed to various factors. It is believed that several virulence factors contribute to fulminant *V.* vulnificus disease,

including lipopolysaccharide, capsular lipopolysaccharide, cytolysin, metalloprotease, and siderophores [7, 19-22].

1.2   Role of iron and other virulence factors for pathogenesis

Iron plays an essential and complex role in the growth and virulence of virtually all microbial pathogens[23]. Iron plays indispensable biochemical roles in oxygen binding, ATP synthesis and DNA metabolism in cell cycle regulation. During infection period it is very substantive for pathogens to acquire iron from their human host. However, almost all the iron present in the human body is sequestered as hemoglobin, myoglobin, or feritin-hemosiderin, and most of the extracellular iron in the body is tightly bound to high-affinity iron-binding compound like the glycoprotein transferrin[24-26]. Hence, there is too little freely available iron in the body to sustain the growth of bacteria[27]. Infection can progress rapidly only in high iron availability conditions. Limiting iron to invading microbes can slow down the course of disease, and allow immune mechanisms more time to clear the infection. In some pathological conditions iron availability increases in the body as intracellular iron is released as the result of cell destruction, resulting in an increase in transferrin iron-saturation levels. As a consequence, host susceptibility to a variety of ferrophilic pathogens, including *V. vulnificus,* increases [25, 28]. Non-transferrin-bound iron (NTBI) which is readily available in the body is required for the initiation of *V. vulnificus* infection under both *in vitro* iron-limited conditions and human *ex vivo* conditions. The excess iron in the body gets deposited in various tissues of the body, particularly in the liver, heart, and endocrine organs [29]. In human and animal studies Hor *et al.* [30]demonstrated that the free levels of iron have markedly increased the growth and lethality of *V. vulnificus*

infection. Many patients especially with chronic liver diseases have a much higher risk some times more than 80% of septicemia and death [1]. According to Blake *et al.* [14], seventy-five percent of infections resulting in septicemia are associated with iron overload, including hemochromatosis, thalassemia, and liver cirrhosis. In majority of the infection, *V. vulnificus* produces siderophores in iron-starvation conditions which are used for the trapping and transporting of iron compounds into the cell [31].

Iron over loaded mice became highly susceptible to *V. vulnificus* infection and their LD_{50} decreased five logs when infected per peritoneum [30], and even up to the 1.1 cells and reduce the time of death after post infection [32]. Additionally, elevated serum iron levels were produced by damaging livers with injection of CCl_4. Similarly, the LD_{50}s were less affected unless the mouse was treated with an additional drug such as cyclophosphamide or D-galactosamine *via* infected with oral route. Mice with or without iron-overloading died when the bacterial concentration in the blood reached 10(5) CFU/mL or above.

The virulence and pathogenesis of *V. vulnificus* is associated with different factors that confer resistance to human innate immunity have been described for biotype 1 strains including polysaccharide capsule that protects bacteria from phagocytosis [33], iron uptake systems (IUSs) used for sequestering iron from host transferrin[34] extracellular hemolysin/cytolysin[21, 35], an elastolytic protease [36], and an endotoxic lipopolysaccharide [37], and resistance to the effects of the bactericidal [38].

Recently, several other genes such as *wbpO, wbpP,* and *wza*, have been described in *V. vulnificus* biotype 1 [39-41]. Moreover, *wbpO genes that*

encodes a putative UDP-*N*-acetyl-D-galactosamine dehydrogenase is essential for LPS biosynthesis, *wbpP gene* encodes a putative UDP-*N*-acetylglucosamine 4-epimerase essential for capsule biosynthesis, and *wza which* encodes a sugar transferase are also needed for capsule biosynthesis. Additionally, *V. vulnificus* biotype 2 serovar E*gne* plays an essential role both in O-antigen biosynthesis of the LPS and in virulence for both humans and eels [41].

1.3   Iron overload and chelation therapy

Patients with chronic anemia such as thalassemia, sickle cell disease and myelodysplastic syndromes can be treated with long term blood transfusion therapy [42-44]. Indeed, many aplastic anaemia patients require repeated blood transfusions to alleviate symptoms of severe anaemia, although these patients can be effectively treated with bone marrow transplantation or immunosuppressive/immunomodulatory therapy. Each unit of blood contains 200-250 mg of iron [43], almost 100 times more than the normal iron absorbed from diet on a single day. After multiple transfusions (10-20 transfusions/ 20-40 RBC units), the body iron will be excessively high as there is lack of proper active mechanisms in the human body to remove extra iron and required iron chelation therapy [45]. Three different iron chelating drugs deferoxamine, deferiprone and deferasirox have been widely used with primary goal of preventing of accumulation of iron reaching harmful levels.

1.4   Antibiotic treatment

The morbidity and mortality of *V. vulnificus* infections can be reduced only by aggressive therapy after suspecting *V. vulnificus* etiology. *V. vulnificus*

infections in humans have been treated with a variety of antimicrobial agents, but the proper selection and administration of antibiotics combined with full medical attention may be crucial in reducing mortality [46]. Clinicians should give more emphasis to the wound site; amputation of the infected limb is sometimes mandatory for survival. The CDC recommends the following antibiotics: (1)Doxycycline (100 mg p.o./i.v., b.i.d. for 7 – 14 days) and a third-generation cephalosporin (*e.g.*, ceftazidime, 1 – 2 g i.v./i.m., t.i.d.); (2) a single-agent regimen with a fluoroquinolone such as levofloxacin, ciprofloxacin, or gatifloxacin; and trimethoprim-sulfamethoxazole combined with an aminoglycoside for children younger than 8 yrs, in whom DX and fluoroquinolones are contraindicated [8].

1.5   Study objectives

Regular blood or red blood cell (RBC) transfusion increases survival time in patients with thalassemia, aplastic to severe anemia, or more complex blood disorders. Iron chelation therapy is required because there is no adequate mechanism for removal of surplus iron deposits in the body, especially in the heart, liver, pancreas, and endocrine glands due to long term transfusion. Following extended use of the parenteral iron chelating drug DFO, growth of ferrophilic organisms such as *V. vulnificus* and *Yesasia enterocolitica* have been reported. However, the potential for the growth and virulence of *V. vulnificus* following treatment with the other two oral iron chelating drugs, DFP and DFS, still remains unknown.

The overall objectives for this research have been to investigate the *in vitro* iron chelation properties of the three commonly prescribed iron chelating

drugs (DFO, DFP, and DFS) for the growth and virulence of *V. vulnificus* organisms, and also to study the potential of combination therapy using CIP and DFS in *V. vulnificus*-susceptible, iron-overloaded patients taking regular blood or RBC transfusions. *In vitro* time-kill studies examining this combination were compared with time-kill kinetics for CIP combined with TG. The purpose of this study was to compare the efficacy of DX p.o. with DX i.p., CIP, CTR, and TG on the survival of mice inoculated with 1×10^8 CFU *V. vulnificus*, and to quantify the DNA copies (and, by extension, bacterial loads) in surviving and non-surviving mice by qPCR to predict the severity of *V. vulnificus* disease.

2.   MATERIALS AND METHODS

2.1   *Vibrio vulnificus* strains used in the study

V. vulnificus (ATCC) 27562 and ATCC 33815 were purchased from American Type Culture Collection and Korean Collection for Type Cultures (KCTC) Canter respectively, and 5 clinical isolates (Table 1) of *V. vulnificus* were selected randomly from patients admitted to Chosun University Hospital and used for various study.

Table 1. *Vibrio vulnificus* strains used in the present study

Strain	Type
ATCC 27562	Type strain
ATCC 33815	Type strain
CUH 4214	Clinical isolate
CUH 4231	Clinical isolate
CUH 4359	Clinical isolate
CUH 4361	Clinical isolate
CUH 4465	Clinical isolate

ATCC, American Type Culture Collection; CUH, Chosun University Hospital

2.2   Media and reagents

The media used were MHB, LB Broth, MHA (Difco Laboratories), LB Plates (LB supplemented 5.0% Agar), top agar (LB containing 0.6 % agar), TSA (Difco ^{TM}, France) plates. Standard CIP powder was obtained from Bayer Health Care, Korea, DFO (Sigma-Aldrich), DFP Ferriprox; Apotex, Toronto, Canada, and DFS (Novartis Pharma, Basel, Switzerland). Iron chelator α, α'

dipyridyl (DD) (Sigma-Aldrich) was added in the medium to obtain iron-poor condition if needed. Ferric chloride (FeCl$_3$) was purchased from Sigma-Aldrich. For the time-kill and minimal inhibitory concentration (MIC) determination, stock solution of the drugs were prepared as 2.048 mg/mL in sterile distilled water however, DFS was dissolved in dimethyl sulfoxide (DMSO, Amresco). Unless otherwise stated, all the chemicals and media were purchased from Sigma-Aldrich.

Iron poor liquid medium (IPLM) was prepared and used for iron chelation experiment with slightly modification of the chemically defined liquid medium of Perry *et al.* [47]and Carniel *et al.* [48]using high purity components containing minimal traces of iron. It contained 180 mM sodium chloride (NaCl), 10 mM ammonium chloride (NH$_4$Cl), 0.4 mM potassium hydrogen phosphate (K$_2$HPO$_4$), 2.5 mM sodium thio sulphate (Na$_2$S$_2$O$_3$), 40 mM morpholinepropanesulfonic acid (C$_7$H$_{15}$NO$_4$S), and 10 mM Tricin (C$_6$H$_{13}$NO$_5$). The pH was adjusted to 7.0 with sodium hydroxide (NaOH) and the medium was heat sterilized at 110°C for 15 min. Fructose (0.1 %) and casamino acids (0.2%; Difco Laboratories) were filter-sterilized before being added to the medium. After preparation of the complete medium (iron deficient medium) the final concentration of iron was fond to be 7.1 ppb by ICP-Atomic Emission Spectrophotometer and was still sufficient to permit the growth of *V. vulnificus*. This iron deficient liquid medium was used to prepare iron-starved bacteria for the synthesis and use of siderophores. Iron-rich IPLM was made by adding 150 μm ferric chloride (FeCl$_3$) (Sigma Aldrich) to the medium 24 hr before use.

2.3   *In vitro* iron chelation experiment

2.3.1   Determination of the MIC of α, α' dipyridyl necessary to inhibit the bacterial growth

Starter culture of *V. vulnificus* ATCC 27562 grown in 10 mL iron poor LB broth (supplemented with 0.2 mM α, α' DD) for 24 hr at 37°C in shaking incubator with 220 rpm was washed twice in 0.9% saline water, and then incubated at a concentration of 10^5 CFU/ml in IPLM alone or in iron-rich IPLM supplemented with 150 µm $FeCl_3$ or with 0.05, 0.1, 0.2, 0.3 and 0.4 mM α, α' DD were incubated at 37° c with 220 rpm. At various time intervals after inoculation, aliquots of the cultures were removed and tenfold dilution in 0.9 % saline was streaked into duplicate onto TSA plates and then bacterial colonies were counted after 24 hr. The minimal concentrations of α, α dipyridyl necessary to inhibit bacterial growth in IPLM were recorded and used in further experiments. Additionally, *V. vulnificus* type strain (ATCC) 27562 was overnight cultured in 10 mL iron-poor LB broth. 0.1 mL of 10^9 CFU/mL bacteria were mixed with 5 mL of melted top agar (0.6 % agar) containing various concentrations of α, α' DD and poured onto LB agar plates containing the same concentration of α, α' DD. The plates were incubated at 37°C and bacterial growth was observed after 24 hr incubation. The minimal inhibitory concentrations of α, α' DD were noted and used in further experiments.

2.3.2   Evaluation of the iron-chelation capacity of deferiprone and deferasirox

V. vulnificus pre-cultured in iron-poor LB broth was inoculated at 10^5 CFU/mL into IPLM alone; IPLM with 0.2 mM α, α' DD; IPLM with DFP, DFO and DFS or with these same concentrations and 150 µm $FeCl_3$. Aliquots

were withdrawn at various times and bacterial growth was monitored by measuring optical density at 600 nm (by spectrophotometer). Similarly, 10-fold dilutions of aliquots were streaked in duplicate onto TSA plates for colony counting. The concentrations of the three iron chelators were chosen to cover the concentrations achieved in human serum (7 to 10 μm for DFO, 70 μm for DFP and 20 μm for DFS).

Furthermore, 10^8 CFUs bacteria pre-cultured in iron-poor LB broth was mixed with 5 mL of melted top agar containing a subinhibitory concentration of α, α'DD (0.3 mM). 20 μl of various concentrations of DFP, DFS and DFO solutions were soaked into 6 mm diameter Whatman filter paper discs (Tokyo Roshi Kaisha, Ltd. Japan) and placed over the solidified top agar. The plates were incubated at 37℃ for 24 hr and then diameter of the zone of growth inhibition surrounding each disk was recorded. The minimal concentrations of DFP and DFS necessary to inhibit bacterial growth were mixed with various concentrations of $FeCl_3$ absorbed on discs and then placed on the top agar. Inhibitory zones were again evaluated after 24 hr.

2.3.3 Kinetics of growth of *V. vulnificus* in the presence of deferiprone, deferasirox and deferoxamine

Five mL of IPLM alone as a control for bacterial growth and IPLM supplemented with 0, 10, 50, 100 μM DFP, DFS and DFO were inoculated with an overnight iron-depleted culture of *V. vulnificus* as described above. These concentrations were chosen in order to cover the range of concentrations achieved in human serum. At 0 to 48 hr post-inoculation, 10-fold dilutions of the bacterial cultures were streaked in triplicate onto TSA plates and CFU were counted after 24 hr.

2.3.4 Growth of various strains of *V. vulnificus* around paper discs absorb into deferiprone, deferasirox and deferoxamine

V. vulnificus strains (ATCC 27562 and 33815, CUH 4359, 4361 and 4465) were grown overnight, mixed with top agar and poured onto iron-poor LB plates contained 0.3 mM α, α' DD as described above. Filter paper discs absorbed 20 µl of various concentrations of DFP, DFS and DFO were placed on the solidified top agar. Plates were incubated at 37°C for 24 hr and the diameters of the zones of bacterial growth surrounding each disk were recorded. The experiments were performed twice.

2.4 Time-kill assays

2.4.1 Minimum inhibitory concentrations

The MICs of CIP, CTR, DX, TG, DFS, and DFP for both type strains and clinical isolates were determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for the micro-broth dilution procedure [49]. The MIC was defined as the lowest concentration of a drug that completely inhibited visible growth of the organism.

2.4.2 *In vitro* time kill evaluation with ciprofloxacin alone and in combination with iron chelator deferasirox

Time-kill studies were performed with CIP alone and in combination with iron chelator DFS to evaluate synergy based on my above *in vi*tro iron cheltation experiment for both ATCC strains and two clinical isolates of CUH [50-52]. Bacteria were diluted to a standardized cell suspension of about 5×10^5 CFU/mL in 50 mL fresh cation adjusted MHB containing 2% NaCl in a 250-mL conical flask, and exposed to different concentration of antibiotic CIP and iron chelator DFS. Growth control was assessed in an extra conical flask

with the same 50 mL MHB but without antibiotic. Each flask was incubated at 37℃ in a shaking incubator at 200 rpm. At 0, 2, 4, 8, 12, 24 and 48 hr of incubation, aliquots of 100 μL bacterial cultures were obtained, spun-down at 3200 g for 15 min, resuspended in fresh MHB to minimize the drug carryover effect and serially diluted 10-fold in MHB and then cultured on duplicate drug free Mueller-Hinton agar plates [53] for overnight incubation in ambient conditions at 37℃ to enumerate CFU/mL [54-55]. Time kill curves were constructed by plotting mean colony counts on log scale versus time with lower limit detection of $2\log_{10}$ CFU/mL. According to the 2009 guidelines of Antimicrobial Agents and Chemotherapy (AAC) and figure 1, synergy was defined as a ≥ 2 $\log_{10}$ decrease (100-fold drop) in the numbers of CFU per mL between the combination and the most efficient agent alone at 24 hr. Indifference and antagonism were defined at 24 hr as $\pm 1-\log_{10}$ kill to <2 compared to the most efficient agent alone and >1 $\log_{10}$ growth compared with the less active agent, respectively [54, 56]. Bactericidal activity of individual drugs alone and drug combinations were defined as ≥ 3 $\log_{10}$ CFU/mL (99.9%) reduction compared to the starting inoculum and to the most active antimicrobial agent at 24 hr respectively [52]. To confirm the results, all the experiments were performed at least in duplicate.
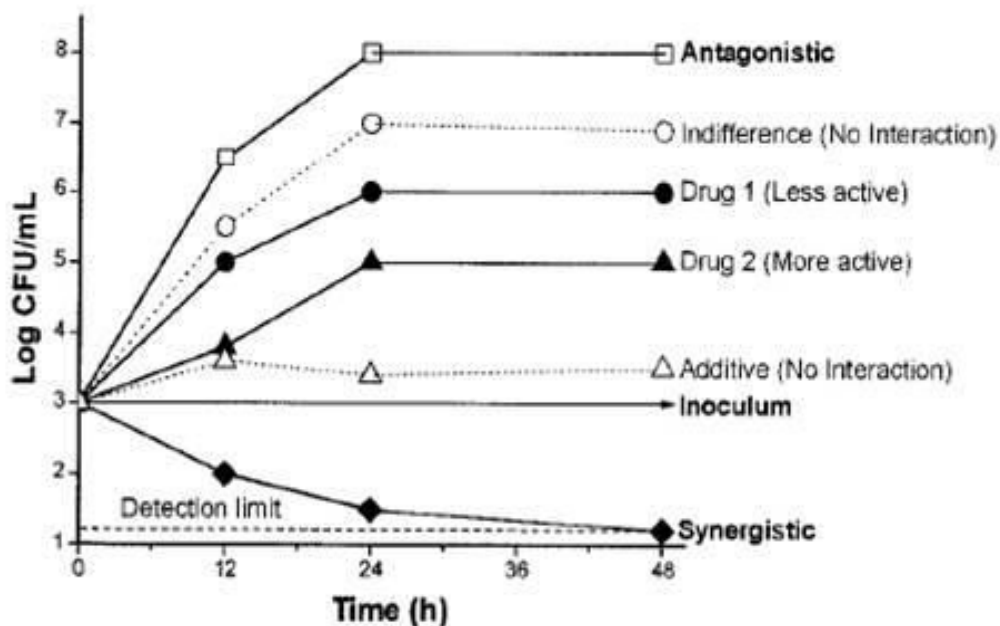
Figure 1. Schematic representation of the criteria used to interpret results from time kill studies of drug-drug interactions. The following criteria are commonly applied: Synergy, a $\geq 2-\log_{10}$ decrease in CFU/mL compared to the most active constituent and starting inoculums; antagonism, a $\geq 2\log_{10}$ increase in CFU/mL compared to the least active drug; additivity, a <2 but >1$-\log_{10}$ decrease in CFU/mL compared to the most active agent; and indifference, a $\pm 1-\log_{10}$ kill to <2$-\log_{10}$ compared to most active efficient agent at 24 hr(Adopted from [57]).

2.4.3 *In vitro* time kill evaluation with chemotherapeutic combinations; ciprofloxacin plus tigecycline

Additionally, time kill experiment was performed with chemotherapeutic combinations of two different drugs having different mode of actions; CIP a bactericidal drug and TG a bacteriostatic drug. To evaluate the *in vitro* activities of the drugs, the sub-MIC (0.75 MIC) of CIP was combining with the same sub-MIC level (0.75 MIC and Supra MIC (1and1.5 MICs) of TG compared individually and in combination as described above. Control growth was assessed in an additional conical flask without drugs and all the experimental procedures were same as above.

2.5 *In vivo* mouse model infection

2.5.1 Animal, bacteria and reagents

Unless noted otherwise, 28-30 gram of 6 week-old male imprinting controlled region (ICR) mice housed under specific pathogenic free/viral antibody-free (SPF/VAF) condition were purchased from ORIENT CHARLES RIVER Technology, South Korea and used in all experiments. The mice were fed a standard laboratory diet and tap water was provided *ad libitum*. Drugs administered were standard injectable formulations of ciprofloxacin (Bayer Vital GmbH, Germany), doxycycline for i.p. (Ben Venue Labs, Inc. Bedford, OH), doxycycline hyclate for p.o. (Sigma-Aldrich), ceftriaxone (Shanghai Roche Pharmaceuticals Ltd, China) and tigecycline (Wyeth, USA), ready to use or diluted in sterile water for injection. For making iron loading condition, mice were i.p. injected with 1.0 mg of filter-sterilized $FeCl_3$ at least 2 hr prior to *V. vulnificus* inoculation. For the mouse model infection, clinical isolate CUH 4214 was selected as being more virulence than both type strains. The animal use protocol was approved by the ethical committee of

the Chosun University, Animal care and use. All animals were treated as humanely as possible.

2.5.2 LD$_{50}$ experiments

For the determination of fifty percent lethal dose (LD$_{50}$), a single colony of *V. vulnificus* grown in TSA plate was picked up and cultured in 30 mL BHI broth and then incubated overnight in ambient condition. Overnight cultured bacteria were diluted 1:20 in fresh BHI broth and cultured for 4−6 hr in same condition unless and otherwise noted. Bacteria were harvested by centrifugation at 6000 x g for 10 min at room temperature and washed twice with PBS and again suspended in PBS to determine the $0.5 \times OD_{600}$ (1 x10^9 CFU/mL) by spectrophotometer. The initial inoculum size was cross checked to match that of a 0.5 McFarland standard by plating in duplicate TSA plates containing 2% NaCl. Bacteria were made 10 fold dilutions (1 x10^9 to 10^2 CFU/mL), and i.p. injected 0.1 mL of the bacterial suspension into the iron loaded mice (n=6) in each group. The control mice were injected only sterile PBS in the same condition.

2.5.3 Animal experiments

An inoculum size of 1x10^8 CFU was chosen for animal experiments to compare the efficacy of different antimicrobial agents for determining the effectiveness of aggressive therapy in humans. One mg of FeCl$_3$ was injected 2 hr prior to bacterial infection *via* i.p. *V. vulnificus* strain grown as above in LD$_{50}$ determination study were prepared for injection (0.1 mL of $0.5O \times D_{600}$) in 90 mice randomly divided into 9 groups. After 1.5 hr of bacterial infection antibiotic therapy was started in mice and monitored for at least 48 hr. Drug dilutions were freshly prepared before use in order to deliver the appropriate dose in a volume of 0.09 − 0.1 mL. All antimicrobial agents were given i.p.

except DX was given both i.p. and p.o. to compare the efficacy of both ways. The doses of antibiotics given was CIP 8 mg/kg body weight at every 12 hr, CTR 50 mg/kg body weight at every 12 hr, TG 7 mg/kg body weight at every 12 hr and DX 6 mg/kg body weight at every 24 hr. Control animals were given 0.1 mL sterile PBS. Antibiotics were given for a total of 24 hr. The mice were monitored in each every 30 min for at least 12 hr and thereafter 24, 36 and 48 hr of *Vibrio* injection. Animals were euthanized when they were moribund even though they were still breathing by considering animal rights. Blood was drawn immediately after euthanized or died by cardiac puncture and stored for −20°C for DNA quantification by real time qPCR.

2.5.4 Quantitative real time PCR

For estimation of bacteria in blood, the maximum amount of blood was collected by cardiac puncture from each sacrificed animal and allocated 200 μL in each eppendrof tube for DNA extraction. Total genomic DNA was extracted from the whole blood as described in the manual supplied with the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Genomic DNAs were stored at −20°C until their use as templates in PCR assays. PCR was performed with a LightCycler (Roche Biochemicals, Mannheim, Germany) using primers and probe for ToxR gene (Table 2) for the whole assay.

The qPCR assay was performed in reaction mixture volume of 20 μL containing 5 μL of template DNA, 1 μL of each of 5 pmol/μL forward and reverse primer (Tox−130 and Tox−200), 1 μL of 2 pmol/μL probe, 4 μL of master mix (reaction buffer, FastStart Taq DNA polymerase, $MgCl_2$, and dNTP with dUTP instead of dTTP) (Roche Diagnostics, Indianapolis), and sterile distilled water as mentioned by Kim *et al.* [58]. The amplification

conditions consisted of pre-incubation (Pre-denaturation) at 95℃ for 10 min and two steps (45 cycles) of denaturation at 95℃ for 10 sec and annealing at 60℃ for 30 s followed by cooling at 40℃ for 30 s. The results were analyzed using Light Cycler Software 4.0 (Roche, Basel, Switzerland). The number of DNA copies obtained from qPCR in different treatment groups was determined by using standard curves plotted against time to obtain the growth kinetics of the bacteria as described by [59].

Table 2. Primer and Probe used for the quantitative PCR

Gene	Primers	Primer sequence	Size (bp)	Reference
ToxR	Tox-130	5'-TGTTCGGTTGAGCGCATTAA-3'		Kim *et al.* (74)
	Tox-200	5'-GCTTCAGAGCTGCGTCATTC-3'		
	Tox-152 Probe	5'-FAM-CGCTCCTGTCAGATTCAACCAACAACG-BHQ1-3'		

2.5.5 Determination of viable *V. vulnificus* cells in real time PCR using ethidium bromide monoazide (EMA).

200 μL thawed mouse blood sample containing *V. vulnificus* cells were treated with 0.25 μg of EMA from a stock solution and tubes placed in the dark at room temperature for 5 min. The tubes were then set into chipped ice, with their lids off, and exposed to the light from a type T halogen lamp (500 W) for 10 min, unless and otherwise indicated, at a distance of 14-15 cm to activate and photolyse the free EMA. EMA, a DNA binding dye was used to differentiate viable and dead cells following implication [60-61], and handled as a carcinogen. Total genomic DNA was extracted from EMA treated whole blood with the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The main

objectives of this study was to determine the number of viable cells of *V. vulnificus* in mouse blood and compared with the cells amplified by qPCR without treating the EMA [62].

2.6   Statistical analysis

All data were analyzed with GraphPad Prism software (Graphpad Software, Inc), Microcal(TM) Origin® version 6.0 software and Microsoft Excel program. For survival analysis, Kaplan−Meier survival curves were analyzed by the log−rank test. An unpaired Student's *t*" test was used to determine significant differences between the groups. All the experiments were repeated at least triplicate. P values of $\leq$ 0.05 or less were considered statistically significant.

3.   RESULTS

3.1   *In vitro* efficacy of Iron chelators deferiprone, deferasirox and deferoxamine on the growth and virulence of *V. vulnificus* infection.

In vitro efficacy of the commonly prescribed three iron chelators DFP, DFS and DFO has been compared to measure their ability of iron chelation to inhibit the growth of pathogenic *V. vulnificus* infections.

3.1.1   MICs of α, α' dipyridyl necessary to inhibit the growth of *V. vulnificus*.

IPLM prepared particularly for this experiment contained minimal traces of iron, but it was still able to promote bacterial growth. I made iron depletion conditions in the medium further by adding various concentration of α, α' DD and growth of *V. vulnificus* was monitored on duplicate streaked plates after 24 hr incubation. Bacterial growth was inversely proportional to the concentration of α, α' DD added to the medium. The minimal concentration of α, α' DD that inhibited bacterial growth was recorded 0.2 mM (Figure 2), and this concentration was used subsequently in further experiments. Additionally, when growth of *V. vulnificus* was examined on iron poor agar plates containing 0.05, 0.1, and 0.2 mM α, α' DD, no bacterial growth was observed beyond 0.3 mM α, α dipyridyl. Hence, 0.3 mM α, α' DD was chosen in further experiments.
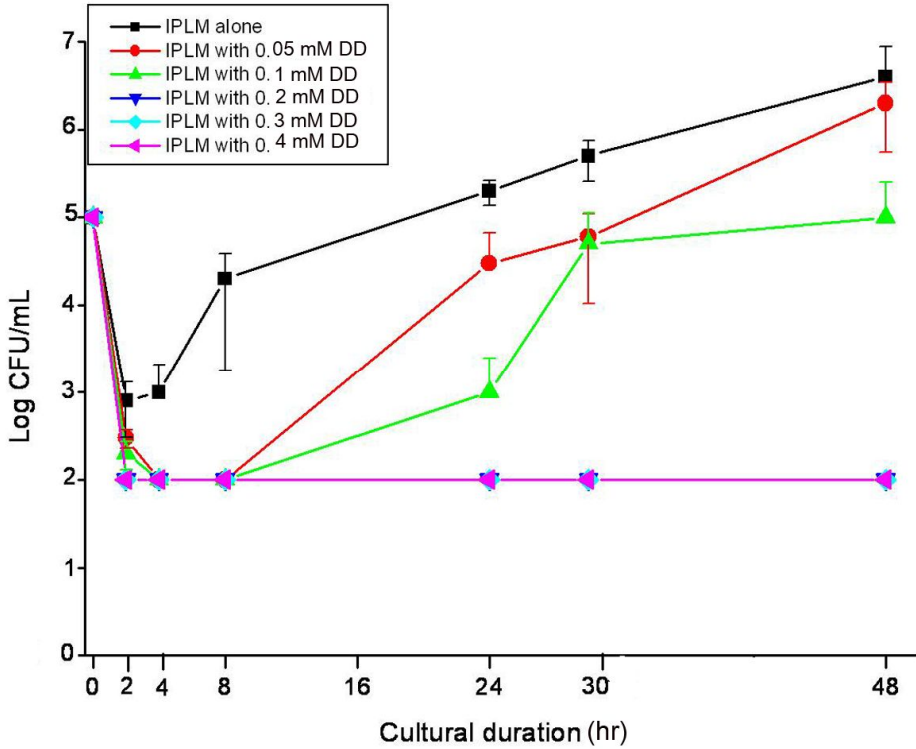
Figure 2. Growth kinetics of *V. vulnificus* in an iron poor liquid medium (IPLM) at various concentrations of α, α' dipyridyl. *V. vulnificus* was grown in IPLM plus 0.05 and 0.1 mM α, α' DD. However, growth of *V. vulnificus* was inhibited in IPLM with 0.2 mM and above concentrations.

3.1.2   Antibacterial activity of deferiprone, deferasirox and deferoxamine

Antibacterial activity of DFP, DFS and DFO by culturing *V. vulnificus* type strain 27562 in IPLM with various concentrations of these chelators. Bacterial growth was inhibited at and above 80 μM DFP and 10 μM DFS. In contrast, no antibacterial activity was observed in DFO even in 40 μM concentration (Figure 3, A−C). Similarly, IPLM containing the MICs of 80 μM DFP and 10 μM DFS was when supplied with 150 μM $FeCl_3$, growth inhibition was abolished. Additionally in agar plates, growth inhibition zone was formed around paper discs impregnated with 10 mM or more DFP, and 5 mM or more DFS, and were proportional to the concentrations of the drugs (Figure 4). However, no growth inhibition zone was formed around paper discs impregnated with concentrations of even 200 mM DFO. The zone of inhibition formed around the Whatman filterpaper discs impregnated with 10 mM DFP and 5 mM DFS, or higher concentrations, were abolished by adding 10 mM $FeCl_3$ to the DFP and DFS (Figure 4). Hence, the inhibition caused by these iron chelators resulted from iron deprivation.
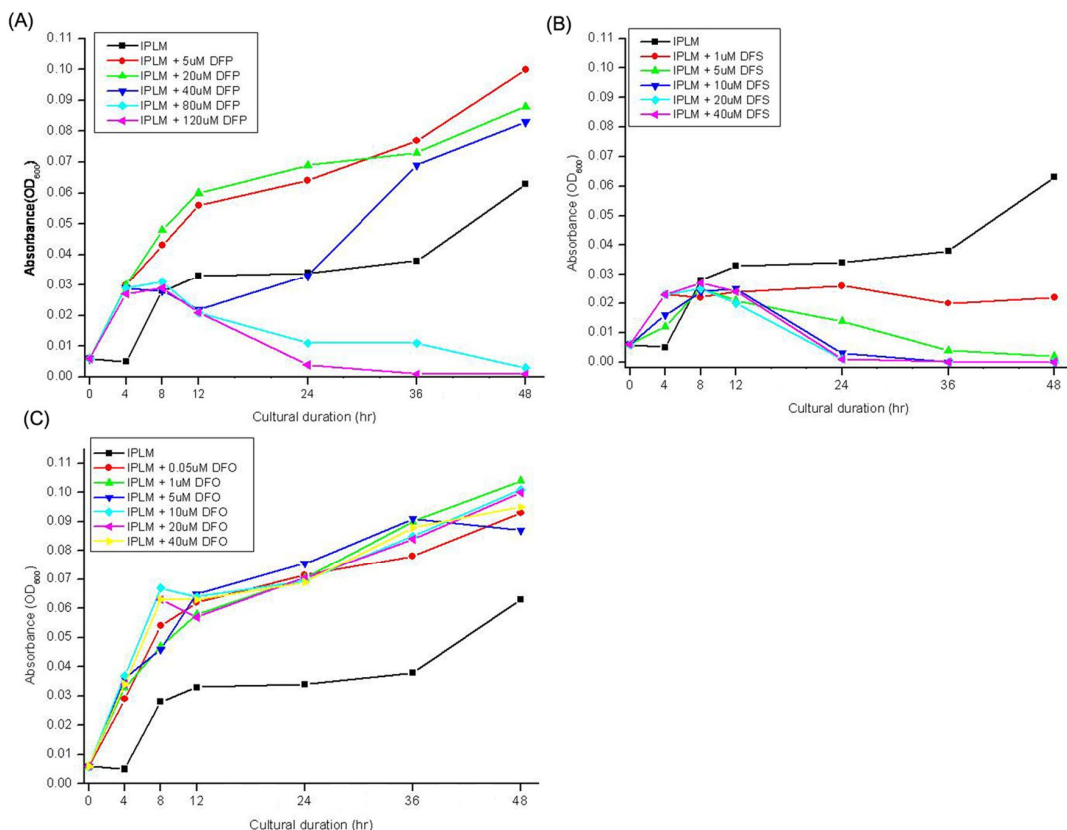
Figure 3. Antibacterial activity of deferiprone, deferasirox and deferoxamine.
Absorbance (OD_{600}) of *V. vulnificus* grown in IPLM alone, and IPLM with
various concentration of DFP (5 to 120 μM, **A**); DFS (0.1 to 40 μM, **B**); and
DFO (0.5 to 40 μM, **C**). or with above concentrations of iron chelators with
150 μM $FeCl_3$. DFP at 80 μM or above and DFS at above 10 μM or above
showed growth inhibition while DFO at even 0.5 to 40 μM concentration
showed no growth inhibition effect. However, the growth inhibition was
abolished by supplying 150 μM iron chloride in both DFP (more than 80 μM)
and DFS (more than 10 μM).
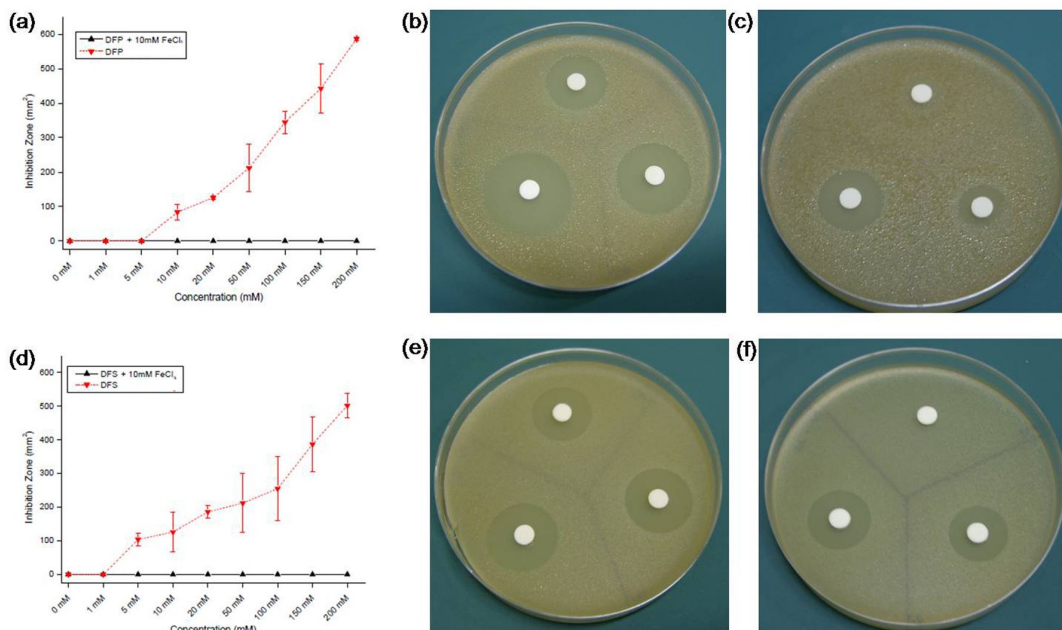
Figure 4. Antibacterial activity of deferiprone and deferasirox

(A)Deferiprone inhibited iron in above 10 μM concentration(red vertical triangle) and it was abolished by adding of just 10 μM $FeCl_3$(upward facing black triangle); (B), zone of inhibition formed by 150, 100 and 50 μM DFP in agar plate; (C), inhibition formed by 20, 10 and no zone of inhibition at 0 μM DFP. Similarly, (D), DFS at above 5 μM concentration formed zone of inhibition (red vertical triangle) and it was abolished by adding 10 μM $FeCl_3$ as in DFP(upward facing black triangle). (E), zone of inhibition by 150, 100 and 50 μM DFS concentrations and (F), inhibition by 20, 10 and no inhibition at 0 μM. Figure (a) and (d) are the mean ± SD of three independent experiments.

3.1.3　Kinetics of *V. vulnificus* in the presence of iron chelators.

The lowest concentration of DFO maximally enhancing the growth of *V. vulnificus* was found to be 10 μM. Additionally, I investigated the potential of DFP and DFS to promote the growth of bacteria under similar conditions, nevertheless none of the concentrations of both oral chelators stimulated growth of the bacteria (Figure 5).

3.1.4　Effects of deferiprone, deferasirox and deferoxamine on various strains of *V. vulnificus* on iron poor agar plates.

The growth promoting effect of three different chelators were investigated by measuring halo of bacterial growth around Whatman filterpaper discs No.1, containing various concentrations of the drugs placed on iron limited agar plates containing 0.3 mM α, α' DD. When various *V. vulnificus* strains were grown on such iron limited agar plates no growth was visible around filter paper discs soaked in solutions containing 0, 0.5 and 1 μM DFO. Zones of growth started to be seen at drug concentrations of 5 μM for ATCC 33815 and the clinical isolate CUH 4359, and at 10 μM for remaining strains (ATCC 27562, CUH 4361 and 4465). Diameters of the growth zones were proportional to the concentration of DFO (Table 3). No growth was visible at any concentration of DFP and DFS.
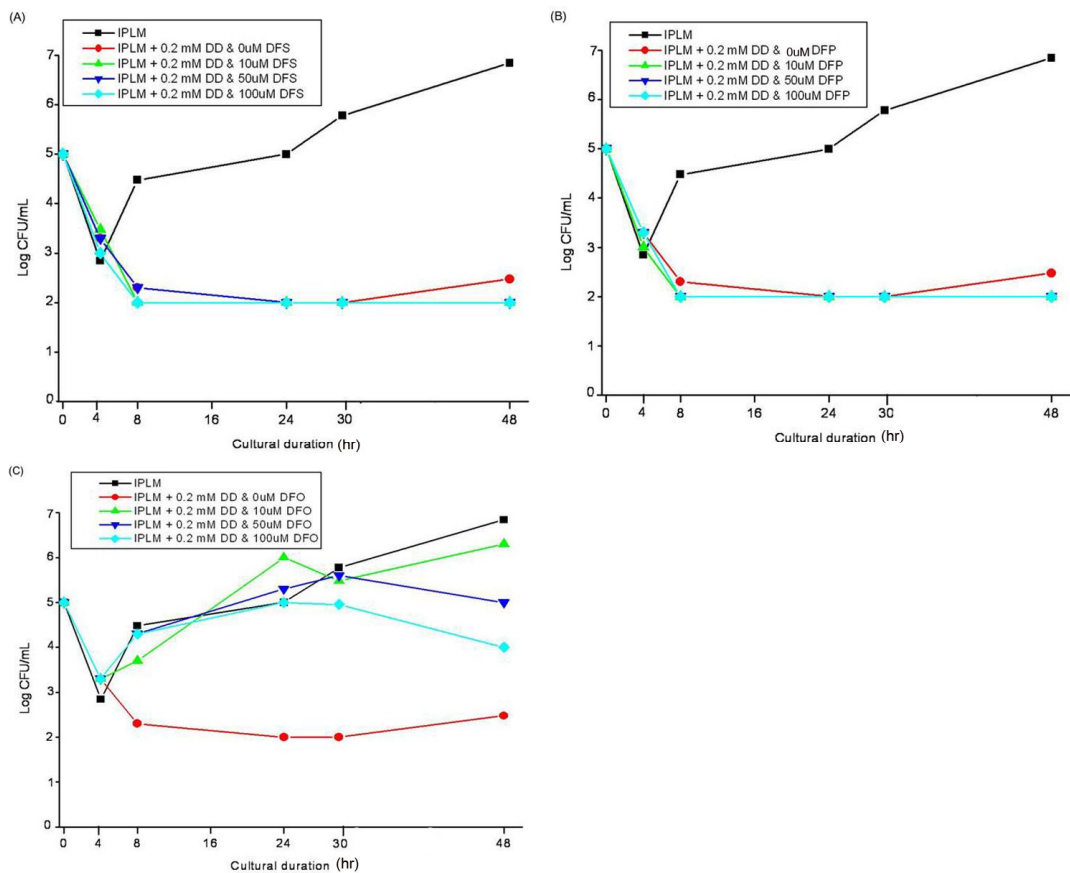
Figure 5. Growth kinetics of *V. vulnificus* growth in iron poor liquid medium plus 0.2 mM α,α′ dipyridyl in the presence of various concentrations (0 to 100 μM) of deferiprone, (A); deferasirox, (B); and (C) deferoxamine.

Table 3. Growth of variuos strains of *V. vulnificus* around filter paper discs impregnated with deferoxamine, deferiprone and deferasirox

Strain	Iron chelators	Diameter (cm) of growth around filter paper disk with indicator concentration.									
		0 µM	0.5 µM	1 µM	5 µM	10 µM	20 µM	50 µM	100 µM	150 µM	200 µM
ATCC 27562	DFO	0	0	0	0	0.8 (0.1)	1.6(0.2)	1.2 (0.4)	1.6(0)	2.2(0.2)	2.8(0.4)
	DFP	0	0	0	0	0	0	0	0	0	0
	DFS	0	0	0	0	0	0	0	0	0	0
ATCC 33815	DFO	0	0	0	0.8(0.1)	1.25(0.25)	1.6(0.4)	1.9(0)	1.95(0.05)	2.1(0.4)	2.25(0.25)
	DFP	0	0	0	0	0	0	0	0	0	0
	DFS	0	0	0	0	0	0	0	0	0	0
CUH 43−59	DFO	0	0	0	0.8(0.1)	1.4 (0)	1.8(0)	1.75(0.35)	2.1 (0.1)	2.15(0.25)	2.4(0.1)
	DFP	0	0	0	0	0	0	0	0	0	0
	DFS	0	0	0	0	0	0	0	0	0	0
CUH 43−61	DFO	0	0	0	0	1.2 (0.1)	1.2(0.2)	1.6(0)	1.8(0.28)	2.2(0.57)	2.2(0.57)
	DFP	0	0	0	0	0	0	0	0	0	0
	DFS	0	0	0	0	0	0	0	0	0	0
CUH 44−65	DFO	0	0	0	0	0	0	1.25(0.25)	1.4(0.1)	2.1(0.1)	1.4(0)
	DFP	0	0	0	0	0	0	0	0	0	0
	DFS	0	0	0	0	0	0	0	0	0	0

DFO, Deferoxamine; DFP, Deferiprone; DFS, Deferasirox and the number in bracket indicates mean standard deviation

Table 4. Susceptibility of two ATCC strains and 5 clinical isolates of *V. vulnificus* to different antimicrobial agents and iron chelators DFS and DFP

Strain No	MIC (μg/mL) for					
	Ciprofloxacin	Doxycycline	Cefotaxime	Tigecycyline	Deferasirox	Defriprone
ATCC 27562	0.0313	0.0313	0.0156	0.0313	8	32
ATCC 33815	0.0625	0.0625	0.125	0.0313	8	32
CUH 42-14	0.0313	0.0313	0.0313	0.016	8	32
CUH 42-31	0.0313	0.0625	0.0313	0.016	8	32
CUH 43-59	0.0313	0.0625	0.0313	0.016	8	32
CUH 43-61	0.0156	0.125	0.0313	0.0313	8	32
CUH 44-65	0.0313	0.125	0.0313	0.0313	8	32

ATCC, American Type Culture Collection; DFP, Deferiprone; DFS, Deferasirox; CUH, Chosun University Hospital

3.2   *In vitro* time-kill activity of ciprofloxacin plus deferasirox

3.2.1 Minimum inhibitory concentrations

The MICs of all isolates including two ATCC and 5 clinical isolates for CIP, CTR, TG, DFP and DFS against the *V. vulnificus* are shown in Table 4. Based on the iron chelation properties of DFS, it has been selected for the time-kill study with alone and in combination with CIP.

3.2.2   Time-kill study

In vitro time-kill studies of CIP plus DFS were performed against four strains including 2 of each, type strains and clinical isolates with various drug concentrations. In time kill study of 0.0225 μg/mL (0.75 MIC) CIP plus 10 μg/mL (1.25 MIC) of DFS, growth inhibition were observed for short time until 2 hr in type strain ATCC 27562. The bacteria then began to regrow slightly and remained constant until 8 hr and thereafter proliferated to an extent level at 36 hr. At 24 hr, the mean reduction in bacterial counts was only 1.2 ± 1.5 CFU/mL and showed indifference(Figure 6, A). However in combination therapy of CIP at 0.0225 μg/mL (0.75 MIC) with 12 μg/mL (1.5 MIC) of DFS, the growth inhibitory effect was continued up to 24 hr with the mean reduction in bacterial counts 2.67 ± 2.6 CFU/mL; and showed synergistic effect (Figure 6, B). In above time kill study, CIP alone at 0.75 MIC seems to be more effective than combination therapy in early hours for the reduction of bacterial counts.

Similarly, in ATCC 33815 strain, combination regimen of CIP at 0.0469 μg/mL (0.75 MIC) plus DFS at 10 μg/mL (1.25 MIC) and CIP at 0.75 MIC plus DFS at 12 μg/mL (1.5 MIC), the growth inhibition was prolonged up to 8 and 12 hr respectively with less killing of bacteria than ciprofloxacin monotherapy at early hours. Afterwards, bacteria in both combination regimens started to

proliferation and the mean reduction of the bacterial counts was 3.1 ± 3.2 in 0.75 MIC CIP plus 1.25 MIC DFS. However, the reduction of bacterial counts was <2 log CFUs/mL as compared to the starting inocula (Figure 6, C) and less signified for synergy. Furthermore, in 0.75 MIC CIP plus 1.5 MIC DFS the mean reduction of bacterial counts was 4.4 ± 5.2 log CFU/mL as compared to single active agent CIP at 24 hr and indicating clear synergistic effect (Figure 6,D).
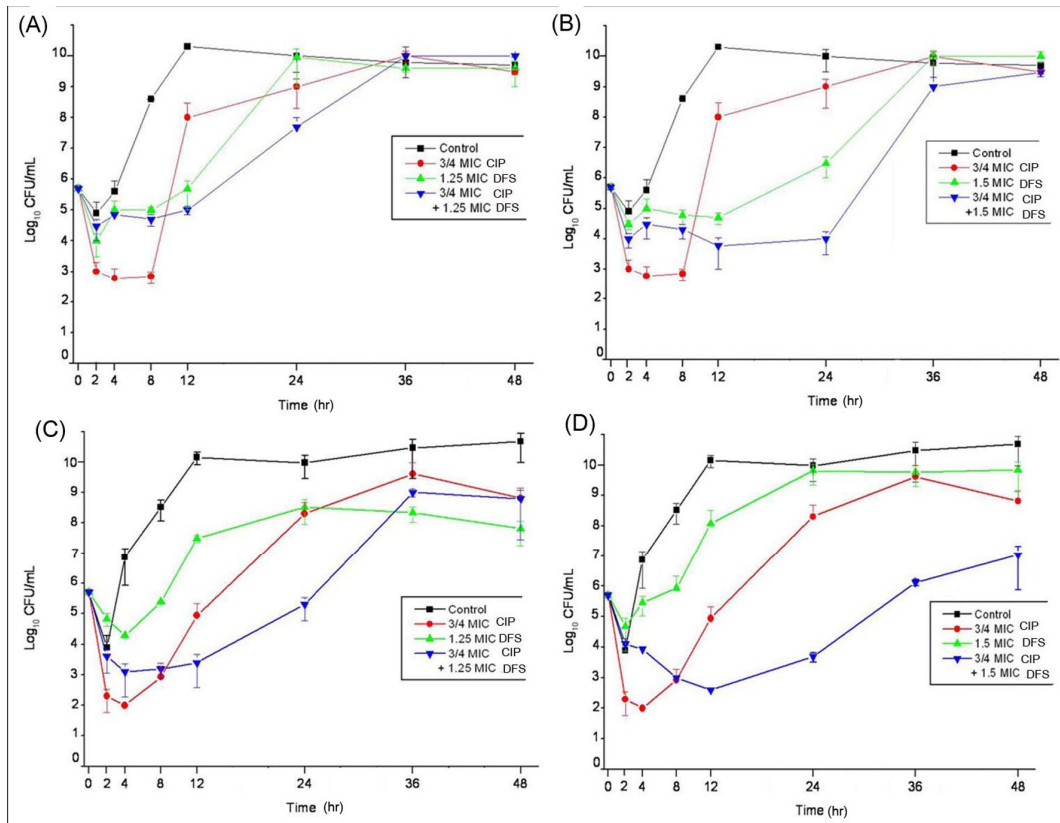
MIC, Minimal inhibitory concentration; CIP, Ciprofloxacin; DFS, Deferasirox

Figure 6. Time-kill kinetics of ciprofloxacin plus deferasirox against *V. vulnificus* type strains ATCC 27562 (A, B) and ATCC 33815 (C, D). The combination of 0.75 MIC of CIP plus 1.5 MIC of DFS reduced bacteria counts by 2 Log_{10} CFU/mL at 24 hr and showed clear synergistic effects (Figure, B and D).

In time-kill study of clinical isolate CHU 4214, it revealed a growth inhibitory effect of CIP at 0.0225 μg/mL (0.75 MIC) up to 4 hr and of DFS at both 10 μg/mL (1.25 MIC) and 12 μg/mL (1.5 MIC) up to 12 hr. However in combination regimen of CIP (0.75 MIC) and DFS (1.25 MIC) the growth inhibitory effect continues till 12 hr and bacteria started to proliferated. At 24 hr, the mean bacterial differences in combination group from a single active agent DFS was just 0.06 ± 0.02 Log CFU/mL and displayed indifferences (Figure 7, A). Accordingly, the growth inhibitory effect of CIP at 0.75 MIC plus 1.5 MIC DFS was continued till 24 hr and the mean reduction of bacterial counts was 2.13 ± 0.3 log CFU/mL as compared to the most active single agent DFS and showed synergistic effect (Figure 7,B).

Similarly, in time kill study of 4231 strain, the growth inhibitory infect was prolonged for 8 hr in 0.0225 μg/mL (0.75 MIC) of CIP and up to 4 and 12 hr in 10 μg/mL (1.25 MIC) and 12 μg/mL (1.5 MIC) of DFS respectively. However in combination therapy of 0.75 MIC of CIP plus 1.25 MIC of DFS the mean reduction of bacterial counts was 0.20 ± 0.2 log CFU/mL as compared to the most active single agent (Figure 7, C). Additionally, the mean reduction of bacterial counts in CIP at 0.75 MIC plus 1.5 MIC of DFS was 2.1 ± 2.1 log CFU/mL at 24 hr. (Figure 7, D).
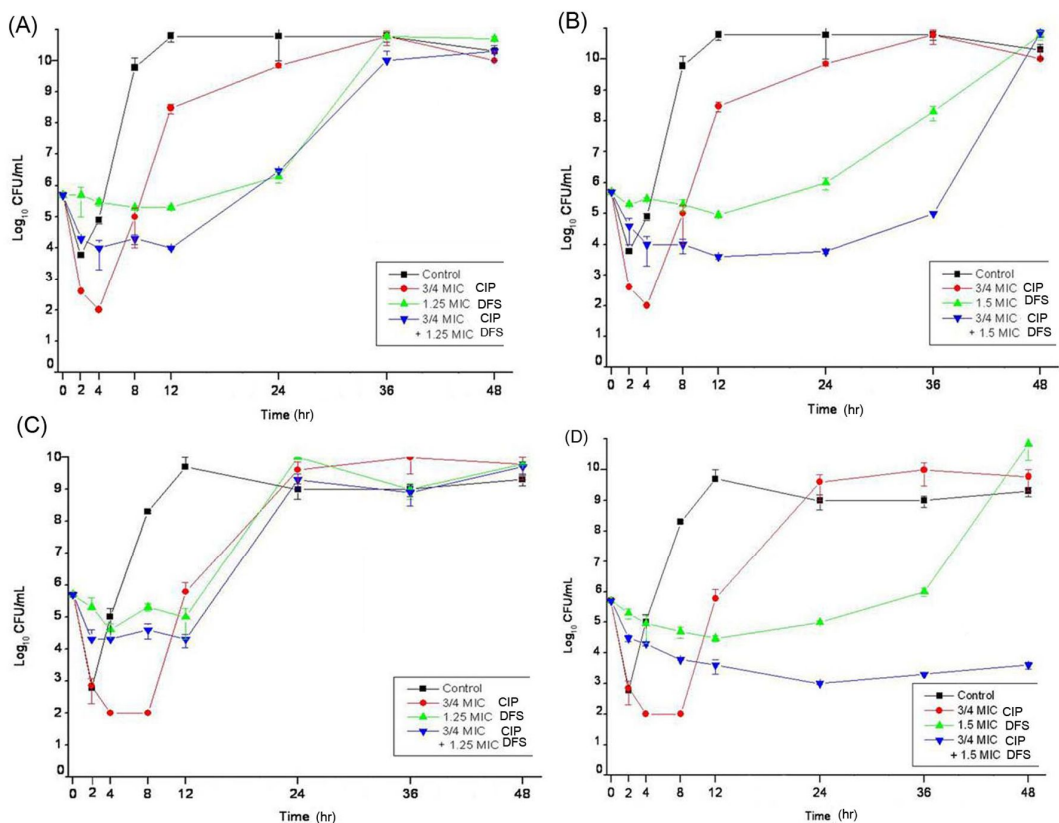
MIC, Minimal inhibitory concentration; CIP, Ciprofloxacin; DFS, Deferasirox

Figure 7. Time-kill kinetics of ciprofloxacin plus deferasirox against clinical isolates CUH 4214 (A, B), and CUH 4231(C, D). The mean reduction of bacterial counts in 0.75 MICs of CIP plus 1.25 MIC of DFS combinations was less than $2Log_{10}$ CFUs/mL as compared to the either most active single agent or with the starting inoculums in both strains (Figure A, and C) and showed indifference. However, the reduction of bacterial counts in 0.75 MICs of CIP plus 1.5 MICs of DFS in both strains was >2 Log_{10} CFUs/mL at 24 hr as compared to either most active single agent deferasirox or with starting inocula and showed clear synergistic effect (Figure B, and D).

3.2.3 *In vitro* time kill studies with ciprofloxacin and tigecycline

Additional time-kill studies with the above strains and one clinical isolate (CUH 4214) were performed to evaluate the combination therapy of CIP and TG and to compare this combination with CIP and DFS. In time-kill studies using *V. vulnificus* ATCC 27562, CIP and TG each at 0.0225 µg/mL (0.75 MIC) inhibited growth for up to 8 hr and 4 hr, respectively. Thereafter, the bacteria regrew and proliferated to an extent approaching the original maximum level. In the combination regimens of CIP and TG each at 0.75 MIC, the reduction of bacterial counts was more significant (at least 3.5 CFU/mL either by comparing with the most active antimicrobial agent CIP or with the starting inoculums) at 24 hr and exhibited synergy (Figure 8, A). In another time-kill study using ATCC 33815, the combination regimen of 0.0469 µg/mL (0.75 MIC) CIP with 0.0225 µg/mL (0.75 MIC) TG gave a more significant reduction of bacterial counts (6.2 CFU/mL by comparing with the single active antimicrobial agent and 3.5 CFU/mL from the starting inoculums) at 24hr and had a synergistic effect (Figure 8, B). Using the clinical isolate CUH 4214, the reduction in bacterial counts following treatment with the combination regimen of 0.0225 µg/mL (0.75 MIC) CIP and 0.012 µg/mL (0.75 MIC) TG was 4.12 ± 4.1 CFU/mL comparing with CIP alone and 0.18 ± 0.5 CFU/mL from the starting inoculum. Synergistic effects were not observed with this combination (Figure 8, C); however, when the same CIP concentration was combined with 0.024 µg/mL (1.5 MIC) TG, the reduction in bacterial counts was more significant (mean 6.46 ± 2.5 CFU/mL compared with the single active antimicrobial agent and 2.3 ± 0.4 CFU/mL from the starting inoculums) and had a pronounced synergistic effect (Figure 8, D).
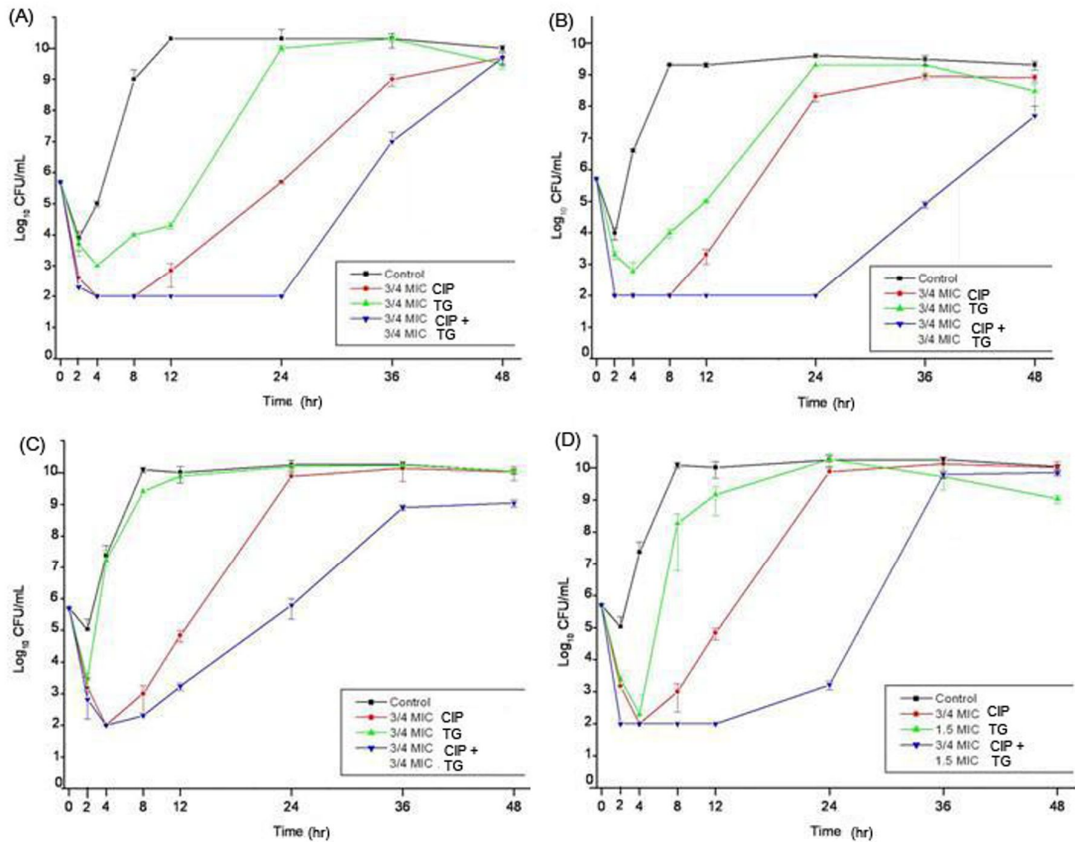
MIC, Minimal inhibitory concentration; CIP, Ciprofloxacin; and TG, Tigecycline

Figure 8. Time-kill kinetics of ciprofloxacin plus tigecycline against *V. vulnificus.* (A), Time kill kinetics of type strains ATCC 27562, (B), ATCC 33815 and (C and D), clinical isolate CUH 4214.

3.3   *In vivo* mouse model infection

A single virulent clinical strain, CUH 4214, was selected for the *in vivo* animal studies. To overcome the high mortality and morbidity of *V. vulnificus* infection, four antibiotics were compared: DX, CIP, TG, and CTR. These were chosen because they are the most commonly prescribed antibiotics for the treatment of *V. vulnificus* infection in humans. When control animals (n = 8) were inoculated with $1x10^8$ CFU bacteria, animals invariably died within the first 4.5 hr.

3.3.1   Selection of clinical strain and LD_{50} study

Iron-overloaded mice, Imprinting Control Region (ICR) were inoculated i.p. with 10-fold serially diluted *V. vulnificus* ($1 \times 10^9 - 1 \times 10^1$ CFU) in sterile PBS. Mice were monitored for at least 24 hr. Mice with rectal temperatures below 33°C were considered moribund and were euthanized. Most mice in the high-dose treatment groups ($1 \times 10^9 - 1 \times 10^6$ CFU) died within 5 hr after inoculation. Most mice in the low-dose treatment groups ($1 \times 10^5 - 1 \times 10^1$), died or were moribund with 24 hr of inoculation, yielding an LD_{50} level <10 CFU.

3.3.2   Monotherpay with ciprofloxacin, ceftriaxone, tigecycline or doxycycline Per Os versus intraperitoneal.

CIP (8 mg/kg body weight), CTR (50 mg/kg body weight), TG (7 mg/kg body weight) or DX both i.p. and p.o. (6 mg/kg body weight) were compared firstly with 10 numbers of mice at each group in above mouse model infection inoculated with high dose *V. vulnificus* (10^8 CFUs) versus control mice without drugs (Figure 9, A). In CTR treated group all mice were died or moribund within 6 hr of *Vibrio* infection and found high number of DNA copies

in blood with no significant differences between control and CTR therapy ($P = 0.30$). In contrast, CIP treated mice were survived for longer duration with 30% survival rate at 48 hr ($P = 0.0002$) showed highly significant and followed by DX i.p. with 20% survival rate ($P = 0.0025$) at 48 hr. The survival rate of TG treated mice was 20 % at 48 hr ($P = 0.032$) while DX p.o. the most commonly ascribed mode for the *V. vulnificus* infection in patients had only 10 % survival rate ($P = 0.25$) (Figure; 9, A; and table 5).

3.3.3 Doxycycline Per Os versus doxycycline intraperitoneal and ciprofloxacin.

Due to high cumulative survival rate of CIP and DX i.p. in above experiment (n=10), I further performed additional experiment by increasing the number of mice (n=21) to compare their efficacy versus DX p.o. In an unavailable condition of DX i.v. in the market, the clinicians need to prescribe p.o. form in combination with extended spectrum cephalosporin for the treatment of *V vulnificus* patients despite no effective survival rate in septicemia cases. However, till date no animal studies were performed for the comparison of the efficacy of p.o. vs i.p. DX administration. In this study, mice survival rate at 48 hr was 19.05 % in DX i.p. group showed highly significant ($P = 0.005$) in contrast to 4.76 % survival rate in DX p.o. treated group at the same time. Indeed, the administration of DX i.p. (6 mg/kg body weight) significantly increased the survival period of *V. vulnificus* infected mice even though survival animals were appeared very sluggish and had ruffled hair up to 12 to 16 hr in contrast to DX p.o. route. Furthermore, CIP treated group had survival rate of 28.57 % showed highly significant versus DX p.o. group ($P=0.0002$) (Figure; 9, B). Indeed, based on the Logrank test, CIP alone showed highly significant as compared to the DX i.p. and other chemotherapeutic drugs TG and CTR.

Table 5. Comparison of the survival curves by log rank test in different antibiotic treated mice infected with *V. vulnificus* clinical isolate CUH 4214 versus control animals treated with PBS.

Treatment group	Chi square	Median survival (hr)	P value
CTR	1.059	3.5	0,3035
DX p.o.	2.360	3.75	0.1245
TG	4.593	6.0	0.0321
DX i.p.	9.126	7.5	0.0025
CIP	13.99	10.5	0.0002

CTR, Ceftriaxone; DX p.o., Doxycycline Per Os; DX i.p., Doxycycline intraperitoneal; TG, Tigecycline; CIP, Ciprofloxacin;
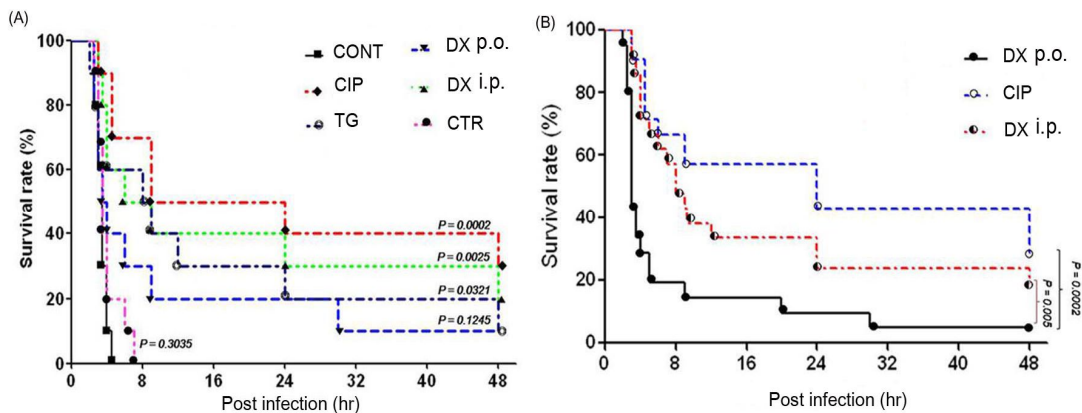
CONT, Control; CIP, Ciprofloxacin; TG, Tigecycline; DX p.o., Doxycycline Per Os; DX i.p., Doxycycline intraperitoneal; and CTR, Ceftriaxone.

Figure 9. Kaplan-Meier survival curve of antibiotic (mono therapy) treated ICR mice (n=10) infected with *V. vulnificus* clinical isolate CUH 4214. (A).CIP showed highly significant followed by DX i.p., TG, DX p.o. and CTR versus control mice given with 0.1 mL PBS instead of antibiotics. Due to high significant values of CIP and DX i.p. these two groups were further compared with DX p.o. in the same high inocula of *V. vulnificus* in iron overloaded mice (n=21) and monitored for 48 hr to draw Kaplan-Meier survival curve. (B). Both CIP and DX i.p. showed highly significant (*P=0.0002* and *0.005* respectively) versus DX p.o.

3.3.4 Combination therapy with ceftriaxone plus doxycycline p.o., ceftriaxone plus doxycycline i.p., ciprofloxacin plus doxycycline i.p., ciprofloxacin plus tigecycline and ciprofloxacin plus ceftriaxone

Interestingly, combination therapies are the most effective therapies for reducing the mortality of *V. vulnificus* infections. During experiment, various combination therapies were compared versus control (Table 6) animals received only PBS and highly used combination therapy DX p.o. plus CTR were compared with other possible combination therapies (Table 3.5). In mouse experiment, iron overloaded ICR mice injected with high inoculum dose of *V. vulnificus* were when treated with DX i.p. plus CTR were survived for more longer time with high significant value versus control mice (*P* = <0.0001), followed by DX i.p. plus CIP (*P = 0.0006*), DX p.o. plus CTR (*P = 0.0014*), CIP plus TG (*P = 0.0171*) and CIP plus CTR (*P = 0.0123*). 50% of the *Vibrio* challenged mice when treated with a single dose DX i.p. plus CTR were survived for more than 48 hr following by 40% survival rate of CIP plus DX i.p. In CIP plus TG 37.5% mice were survived for more than 48 hr. The *in vitro* time kill study of CIP plus TG showed synergism; however, mice survival in CIP plus TG was less significant than CTR plus DX i.p. Furthermore, DX p.o. plus CTR and CIP plus CTR treated groups all mice were died before the 48 hr (Figure 10, A).

Furthermore, Kaplan−Meier survival curve of various combination therapies were compared versus DX p.o. plus CTR. Among these combinations, DX i.p. plus CTR showed highly significant *(P = 0.01)* following by CIP plus DX i.p. *(P = 0.057)*, CIP plus TG *(P = 0.072)* and CIP plus CTR *(P = 0.98)* (Figure 10, B). During my experiment, combination therapy treated survival mice at 48 hr had survived longer time without any symptoms of diseases, however most of the monotherapy treated mice survived at 48 hr died within 1−2 weeks.

Table 6. Comparison of the survival curves by log rank test in combination therapy of mice infected with *V. vulnificus* infection versus control mice treated with PBS.

Treatment groups	Chi square	Median survival(hr)	P value
CIP + TG	5.687	7.5	0.0171
CIP + CTR	6.260	4.5	0.0123
CTR + DX p.o.	10.20	10	0.0014
CIP + DX i.p.	11.79	24	0.0006
CTR + DX i.p.	15.82	36	<0.0001

CIP, Ciprofloxacin; TG, Tigecycline; CTR, Ceftriaxone; DX p.o., Doxycycline Per Os; DX i.p., Doxycycline intraperitoneal

Table 7. Comparison of the survival curves by log rank test in combination therapy of mice infected with *V. vulnificus* clinical isolate CUH 4214 versus ceftriaxone plus coxycycline Per Os.

Treatment groups	Chi square	Median survival (hr)	P value
CIP + CTR	0.00033	4.5	0.98
CIP + TG	0.1238	7.5	0.72
CIP + DX i.p.	3.634	24	0.057
CTR + DX i.p.	6.223	36	0.01

CIP, Ciprofloxacin; TG, Tigecycline; CTR, Ceftriaxone; DX p.o., Doxycycline Per Os; DX i.p., Doxycycline intraperitoneal
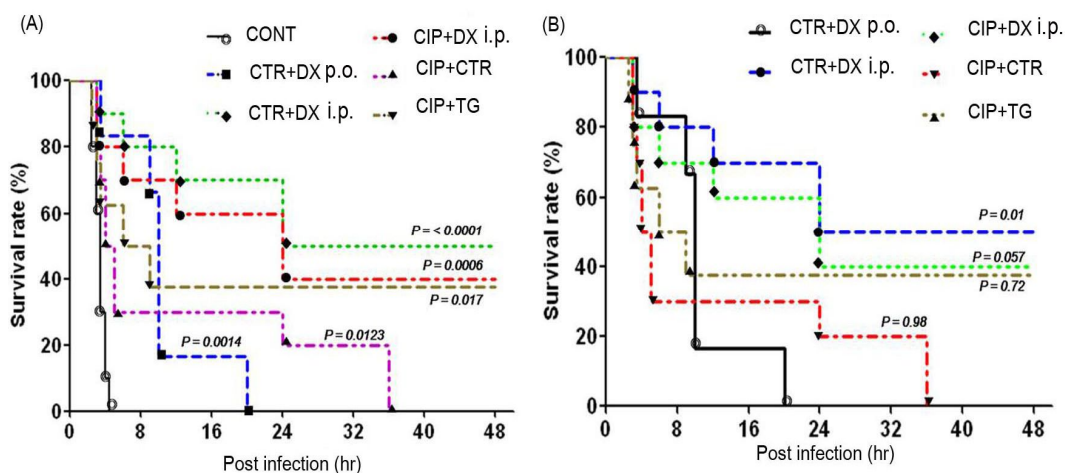
Note: CONT, Control; CIP, Ciprofloxacin; DX p.o., Doxycycline Per Os; DX i.p., Doxycycline intraperitoneal; CTR, Ceftriaxone; TG, Tigecycline

Figure 10. Kaplan-Meier survival curve of antibiotic combination therapy treated ICR mice infected with *V. vulnificus* clinical isolate CUH 4214. (A). CTR plus DX i.p. showed highly significant following by CIP plus DX i.p., CIP plus TG, CIP plus CTR and DX p.o. plus CTR versus control mice given PBS instead of antibiotics. (B). CTR plus DX i.p. only showed statistically significant versus CTR plus DX p.o. while rest of the combinations did not showed statistically significant.

3.3.5   Quantitative PCR assay for *V. vulnificus* detection and mortality

A highly sensitive quantitative PCR assay was used to quantify the bacterial DNA copy number more accurately in blood in order to monitor the course of the disease and to compare the effectiveness of the antibiotics used in different treatment groups. Mouse after given antibiotic was monitored regularly in every 30 min for first 12 hr and thereafter at 24, 36 and 48 hr to draw the blood immediately when they died or moribund. Additionally, blood was also drawn by cardiac puncture after sacrificing at 12 and 24 hr survival mice to identify the bacterial load in survival mice after post infection and chemotherapy treatment. During qPCR, plasmid DNA of 5×10^8 copies/μL was used as template and standard curve was drawn by serially diluted the template DNA in nuclease free sterile DW from 5×10^8 to the 5×10^0 copies/μL (Figure 11, A). Genomic DNA extracted from mouse blood was used for the quantification of bacterial DNA copies/μL of all treatment groups. In iron overloaded mice, the LD_{50} was determined as below 10 CFUs. The number of DNA copies of early died mice in all antibiotic treated group contains high load of DNA (1×10^5 to 1×10^7 copies/μL), and it reduced by increasing of the survival length of the mice. However, the number of DNA copies in DX p.o. treated died mice at the 3 to 12 hr ranges (4.21×10^7 to 4.34×10^6 copies/μL) in contrast to DX i.p. treated died mice having (1.28×10^7 to 4.87×10^4 copies/μL) (Figure 11, B). Additionally, the DNA copies in CTR treated mice were very high as control treated mice and ranges from 1.76×10^7 to 3.19×10^6 copies/μL with Cq values of $14.75-17.75$ (Figure 11, C).
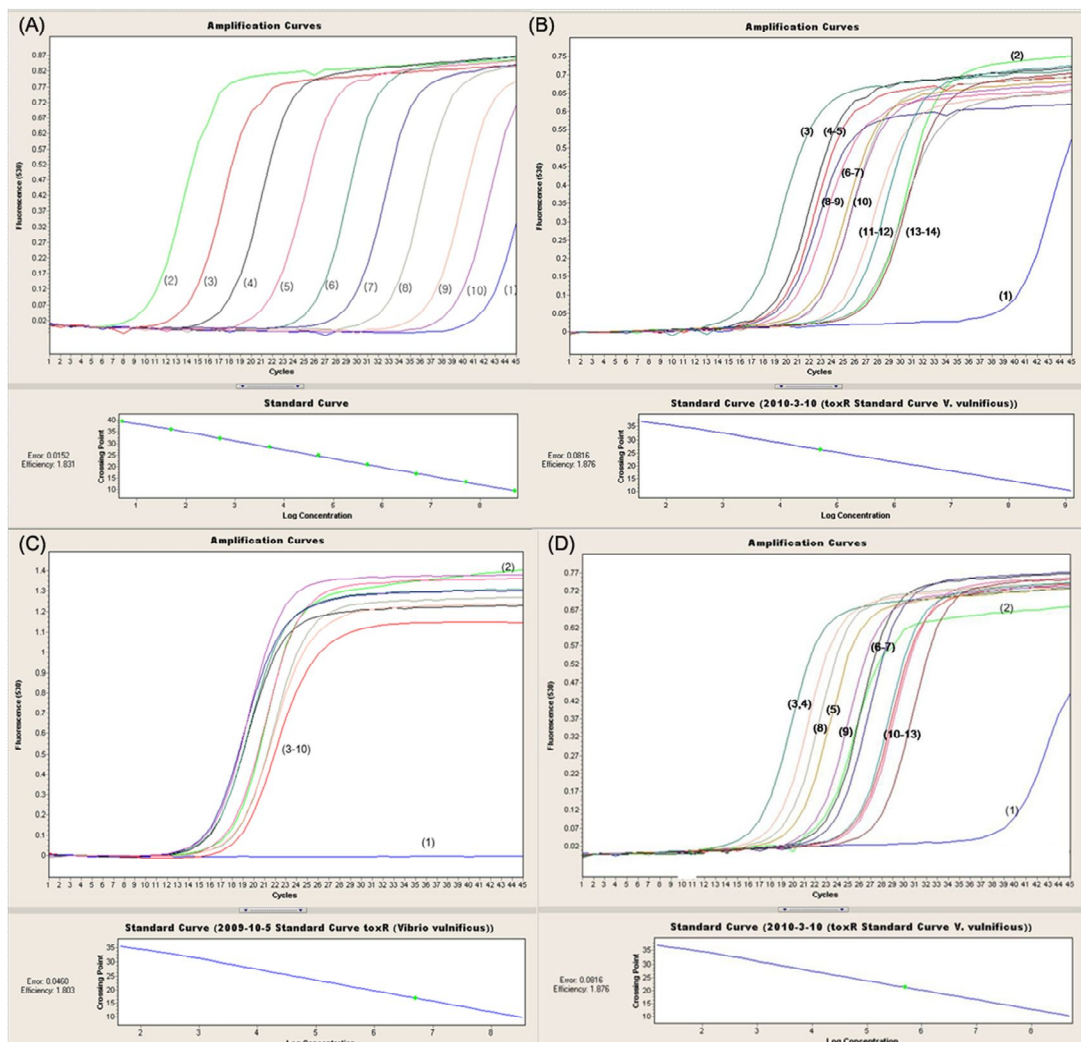
Figure 11. Quantitative real time amplification curves. (1), negative control (sterile DW); (2), known plasmid DNA was used as the template numbers in all figures. (A), qPCR standard curves of 5×10^8 to 5×10^0 copies/μL (3−10 10 fold serially diluted). The amplification plot taken from the base shows a good linearity across the entire concentration range. The E value (base of the exponential amplification function) of 1.83 (corresponding to amplification efficiency) falls within the specifications and this known sample was subsequently used to identify the DNA copies from mouse blood; (B),

amplification plot (curves) in DX p.o. and DX i.p. treated mice. Numbers 3 to 7 showed amplification curves from DX p.o. treated died mice and 8-14 amplification curves from DX i.p. treated died mice; (C), amplification curves of DNA from CTR treated died mice where all mice were died within 6 hr; and (D), amplification curves of DNA from CTR plus DX i.p. versus CTR plus DX p.o treated died mice at various time intervals (3 to 12 hr). Number 3-7 amplification curves of DX p.o. plus CTR treated group while 8-13 are the amplification curves from DX i.p. plus CTR treated group.

Furthermore, the number of DNA copies in CTR plus DX p.o. treated died mice were ranges in 2.86×10^6 to 4.16×10^4 copies/µL in contrast to 3.9×10^5 to 5.33×10^4 copies/µL range of CTR plus DX i.p. treated died mice of the same time (Figure 11, D). However, both the monotherapy and combination therapy treated group did not show statistically significant.

Furthermore, the DNA load in all mortality case mice was at least 1×10^4 copies/µL at 12 hr in contrast to 10^2 copies/µL range in survival case at the same time (Figure 12, A). Furthermore, the qPCR result showed the number of DNA copies in survival mice at 12 and 24 hr in both CIP and DX i.p. treated mice were range in 10^2 copies/µL in contrast to 10^4 copies/µL in DX p.o. treated mice survived for 12 hr. Similarly, in combination therapy of survival group treated with CTR plus DX i.p., the DNA copies were 10^2 copies/µL at both 12 and 24 hr in contrast to CIP plus DX i.p. treated mice of 10^3 copies/µL. Additionally, in CTR plus DX p.o. treated mice, the DNA copies at 12 hr was almost same as DX p.o. monotherapy (Figure 12, B).

45
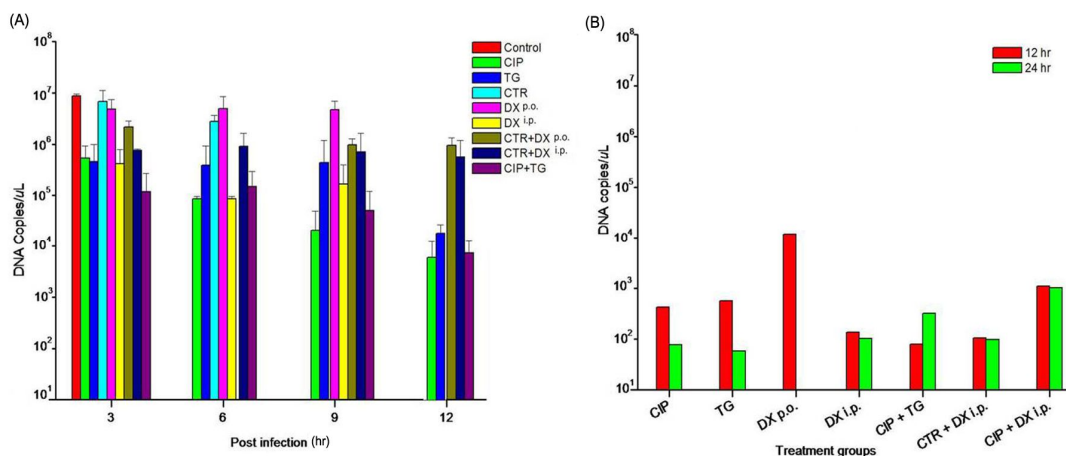
CIP, Ciprofloxacin; TG, Tigecycline; CTR, Ceftriaxone; DX, Doxycycline; p.o.; Per Os, i.p., Intraperitoneal.

Figure 12. Number of DNA copies/μL in non-survival and survival mice after antibiotic treatment. (**A**), number of DNA copies/μL in control (PBS treated instead of antibiotic) and various drug treated mice died at 3, 6, 9 and 12 hr after post infection. (**B**) number of DNA copies/μL in survival mice at 12 and 24 hr in various monotherapy and combination therapy. Mice survived at 12 and 24 hr were sacrificed and blood was drawn for qPCR. CIP and TG alone seems equally better as combination therapy in terms of DNA load at 24 hr survival mice. However, the combination therapy treated mice with CTR plus DX i.p. had minimal DNA copy numbers at both 12 and 24 hr survival mice and this group had more survival percentage than monotherapy.

3.3.6 Quantitative PCR assay and determination of viable *V. vulnificus* cells

To determine the viable cell, mouse blood was treated with EMA prior to DNA extraction. EMA was used to distinguish only viable cells and handled as a carcinogen in though out the study. The mechanism of using EMA was for the selective penetration of dye into the cells with damaged membrane of dead cells. In DX p.o. and DX i.p. treated mice, difference in DNA copy number in with or without treatment of EMA were almost $1\log_{10}$ difference showed statistically no significant difference between two groups. It might be due to the early drawing blood after they died or moribund and freezes immediately at $-70°C$ and DNA was extracted within 24 hr of mouse experiments (Figure 13).
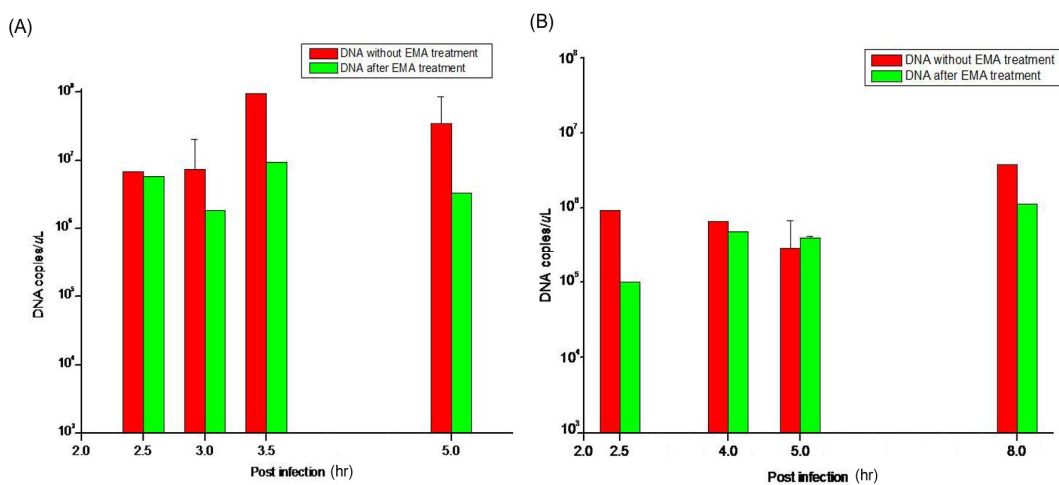
Figure 13. Determination of viable cells from doxycycline Per Os and doxycycline intraperitoneal treated ICR mice. (A), DNA copies in DX p.o. treated died mice at 2.5 to 5 hr of post infection showed high copy value (1×10^{6} to 1×10^{8} copies/μL); (B), number of DNA in DX i.p. treated died mice at 2.5 to 8 hr post infection showed maximum difference of $1\log_{10}$ at early 2.5 hr. In both group there was no statistically significant difference in with or without treatment of EMA.

4.   DISCUSSION

V. vulnificus is a halophilic, Gram-negative bacillus that causes life-threatening skin and soft tissue infections. Elevated serum iron levels may play an important role in the pathogenesis of *V. vulnificus* infections [25]. Concentrated *V. vulnificus* exerts pronounced cytotoxicity in cell culture and infected animals. Under conditions of iron overload, the production of tumor necrosis factor alpha (TNF-α) is stimulated. This cytokine is a major mediator of septic shock in mice infected with *V. vulnificus*. TNF-α production is probably induced by the bacterial endotoxin; however, TNF-α dependent migration of neutrophils also appears to be suppressed during infection[63]. Under conditions of iron overload, circulating numbers of *V. vulnificus* in the blood accelerated alarmingly to a lethal level and lowered the activity of immune cells including neutrophils. Virulence factors other than TNF-α that also are stimulated by the endotoxin may play a crucial role in the enhanced destruction of host cells, including the immune cells, during infection.

V. vulnificus required a higher level of nontransferrin-bound iron (NTBI) than other pathogens for the initiation of growth. This bacterium also produced siderophores at lower levels and used transferrin-bound iron (TBI) less efficiently than other bacterial species [28]. The standard iron chelating agent, DFO, a hydroxamate siderophore derived from *Streptomyces* species, can accelerate the *in vitro* growth of many ferrophilic organisms such as *V. vulnificus, Yersinia enterocolitica*, and *Mucorales*. These organisms bind specifically to DFO-iron complexes and strip iron from the chelator via an energy-dependent reductive process that facilitates iron uptake [64]. DFO is a widely prescribed parenteral drug used as an iron chelating agent for the

treatment of thalassemia. However, DFO may increase the risk of fatal infections by promoting the growth of *Y. enterocolitica* by permitting efficient iron uptake via specific receptors such as the outer membrane protein FoxA [65]. Similarly, *V. vulnificus* can use DFO for efficient iron uptake via the outer membrane receptor DesA, encoded by the *desA* gene, under iron-limited *in vitro* and *ex vivo* conditions [64]. *V. vulnificus* is known to require higher levels of available iron for growth than other pathogenic bacteria because it produces less siderophores than *Escherichia coli* and *Staphylococcus aureus* [28]. *V. vulnificus* produces both the hydroxamate and vulnibactin types of siderophores. However, hydroxamate siderophores alleviated the growth of *V. vulnificus* under iron-limited conditions [31], and DFO-mediated iron chelation plays an important role in *V. vulnificus* growth initiation [64]. In some patients treated with an iron chelator such as DFO in whom yersiniosis or mucormycosis was promoted, the drug itself may have enhanced the growth of a microbial or neoplastic cell invader by acting as a siderophore instead of assisting the host [67].

V. vulnificus sepsis that developed during DFO therapy for transfusional iron overload due to secondary hemochromatosis of myelodysplastic syndrome was also successfully treated with adjuvant DFS in combination with CIP [68]. Additionally, Kim *et al* [28] suggested that iron chelation therapy may be an effective means of preventing the growth of *V. vulnificus* in susceptible patients. In this study, combinations of the three chelators, including DFS and DFP with DFO, were compared to characterize the *in vitro* iron chelation properties of these drugs. Results suggest that the growth of *V. vulnificus* was stimulated by DFO, whereas the two oral chelators, DFP and DFS, had an inhibitory effect on the *V. vulnificus* growth. No bacterial regrowth was observed for $\geq$ 48 hr in the presence of $\geq$ 80 μM DFP and $\geq$ 5 μM DFS.

DFO-treated patients with evidence of iron overload, such as those with hemochromatosis or thalassemia, are known to be susceptible to fulminant *Y. enterocolitica* septicemia and *Mucorales spp.* [65, 69]. They also are susceptible to *V. vulnificus* infection [68]. The *in vitro* data presented here demonstrate that DFS or DFP potentially offer greater clinical application than DFO for patients in need of iron chelation therapy. This is especially true of patients residing in areas where the habit of eating raw seafood or being exposed to contaminated seawater is more prevalent.

The concentrations of DFP, DFO, and DFS achieved in human sera are known to be 70 μM, 7 – 10 μM, and 20 μM, respectively, when treating iron overload in blood transfusion patients [65, 70]. In the iron chelation experiments described here, 5 – 120 μM DFP and 1 – 100 μM DFS were used to simulate the usual serum concentration of iron chelators in iron-overloaded patients. Results suggested that iron chelation therapy that maintains serum concentrations of more than 5 μg/mL DFS or 80 μg/mL DFP should be adequate for preventing *V. vulnificus* infection in iron-overload patients; however, further *in vivo* experiments are needed to validate this. Hence, DFS and DFP are not only useful for the treatment of iron overload, but also function as antimicrobial agents with the potential to act synergistically with various antibiotics [71].

For further confirmation, *in vitro* time-kill studies were performed to determine the efficacy of the combination of CIP and DFS versus CIP monotherapy against *V. vulnificus* infection. When 0.75 MIC CIP was combined with 1.5 MIC DFS, the inhibition of bacterial growth *in vitro* persisted in all strains for ≥ 24 hr, and the combination was superior to CIP

alone. The antimicrobial effect of DFS may be ascribed to its ability to deplete iron that would otherwise be used for bacterial growth.

The combination of an iron chelator plus an antibiotic creates a novel form of synergism. It is possible that the quinolone antibiotic interferes with DNA gyrase and that the microbe consequently becomes more sensitive to iron deprivation [71]. Based on the time-kill studies described here, it would appear that CIP and DFS are an active combination against *V. vulnificus*. Even though CIP and DFS have a synergistic effect against *V. vulnificus*, it should be noted that CIP alone appeared to be more active at 2 to 8 hr than the combination treatment. In other experiments, higher CIP concentrations were found to be more effective at inhibiting the proliferation of microorganisms. Therefore, a high-dose CIP treatment may be required to overcome the suboptimal effect of CIP and DFS at early times. It will be important to test for *in vivo* synergism of CIP and DFS to establish the clinical relevance of these findings.

In microbial infection, as in cancer therapy, much attention has been paid to the discovery of synergistic drug interactions. Drugs from different classes that act on distinct cellular pathways can potentiate each other either additively or synergistically when used in combination [72]. For example, the antigrowth effect of antibiotics from a class of DNA-synthesis inhibitors working neither additively not synergistically, but subtractively and exhibited hyper-antagonistic characteristics class when combined with drugs from a class, of protein synthesis inhibitors [73]. Jawetz and Gunnison [74, 75] postulated that "the bacteriostatic antibiotics may antagonize the action of bactericidal drugs, and they may be synergistic when bactericidal drugs used together". Since then, combination therapy has become a fundamental

principle of antibiotic therapy [76]. The combination of CIP, a bactericidal drug, and DX, a tetracycline group of antibiotics having bacteriostatic properties, was the most active combination, as assessed *in vitro* using *Brucella* strains [77]. Similarly, DX combined with CTR or cefixime (bactericidal drugs) is considered the first-line empiric therapy in patients with urethritis [78].

Many *in vitro* and *in vivo* combinations of antimicrobial agents have been studied for treating *V. vulnificus* infections [4, 7, 50, 79]. Among various combination therapies, only a few are effective in reducing patient mortality. *In vitro* and *in vivo* studies by Chuang *et al* [51, 79] suggested that combination therapy of cefotaxime and minocycline was more advantageous than either antibiotic alone for the treatment of severe experimental murine *V. vulnificus* infections. Additionally, fluoroquinolones alone have been shown to be as effective as the combination of cefotaxime plus minocycline *in vitro* and *in vivo* [4].

This research investigated the *in vitro* combination therapy of CIP with TG, a newly approved glycylcycline class of antibiotics derived from tetracyclines. TG exhibits remarkable *in vitro* activity against a wide variety of Gram-positive and Gram-negative bacteria, including multidrug resistance (MDR) strains [80]. TG was preferred because the current available data suggest that this drug may play an important future role as a monotherapy alternative to broad-spectrum antibiotics, such as advanced-generation cephalosporins, carbapenems, fluoroquinolones, piperacillin/tazobactam and some Gram-positive-directed agents [80]. Furthermore, TG has been investigated in combination with a large variety of antimicrobials against a wide range of susceptible and multiresistant Gram-negative and Gram-positive bacteria

[81]. Earlier, Liu *et al* [82] described excellent *in vitro* activity of TG against clinical isolates of *Aeromonas, Vibrio*, and nontyphoidal *Salmonella* species (NTS). TG alone or in combination with other drugs may be a promising option for the treatment of *Vibrio* species infections owing to its low MIC against *V. vulnificus* and *V. parahaemolytics* isolates. Indeed, TG is safe as its high concentrations for treating skin and soft tissue infections [83]. Perhaps most promising is the fact that TG is not affected by common tetracycline resistance mechanisms including tetracycline-specific efflux pumps and ribosomal protection.

Combination of TG *in vitro* with other antimicrobial agents produces primarily an indifferent response (neither synergy nor antagonism). However, it produces synergy when combined with rifampicin against 64 – 100% of *Enterococcus* spp., *Streptococcus pneumonia*, *Enterobacter* spp., and *Brucella melitensis* [81]. Vouillamoz *et al* [84] when tested 88 isolated bacteria (22 Gram-positive and 55 Gram-negative) with 16 different antibiotics in combination with TG by chequerboard methods and time-kill assays showed one synergism between TG and CIP in P. aeruginosa.

In the present studies, time-kill analyses using CIP and TG suggested synergism in three *V. vulnificus* strains, including two ATCC strains and one clinical isolate. When 0.75 MIC CIP was combined with 0.75 MIC TG, the reductions in bacterial counts in both ATCC strains were > $3\log_{10}$ CFU/mL and displayed synergism. However, in clinical isolates, the reduction of bacterial counts at same drug concentrations exhibited indifference and required additional TG to display synergism. Specifically, 0.75 MIC CIP combined with 1.5 MIC TG synergistically reduced bacterial counts ($2.3\pm$ 0.4) CFU/mL compared to the starting inoculums) at 24 hr in clinical isolates.

53

The pros and cons of Jawetz and Gunnison's [74, 85] chemotherapeutic interactions have been found especially in the treatment of some invasive Gram-positive and Gram-negative pathogens. *V. vulnificus* might be the exception in which combinations of bacterial and bacteriostatic drugs is superior to combinations of bacteriostatic drugs for the treatment of septicemia. Even though *V. vulnificus* infections have been treated with a variety of antibiotics and are highly susceptible by *in vitro* studies, no clear conclusions can be made concerning the relative efficacy of individual antibiotics. Toward this end, *in vivo* mouse models of infection were used to study the efficacy of highly prescribed antibiotics in high-dose *Vibrio*-injected ICR mice. *In vitro* and *in vivo* efficacy of several antimicrobial agents showed considerable discrepancies in terms of the bacterial response. This might be due to poor perfusion and consequently poor penetration into the edematous focus of infection [46]. Hence, a high *in vitro* activity for a given antimicrobial agent may not be equally effective in patients infected with *V. vulnificus*. The *in vivo* efficacy of antibiotics used in this study was evaluated by comparing the survival curve percentage between the control and antibiotic-treated mice. The choice of antimicrobial agents and their respective doses was based on previous *in vivo* studies in mice and humans. Antibiotics were given in 24 hr doses, and mice were monitored for at least 48 hr to plot the survival curve.

CIP alone appears to be effective in monotherapy ($P = 0.0002$) for prolonging survival time in mice challenged with high-dose *V. vulnificus* followed by DX i.p. ($P = 0.0025$) and TG ($P = 0.0032$). DX p.o. and CTR alone were the least effective treatments compared with PBS-treated control animals. In combination therapy, a single dose of DX i.p. and CTR was sufficient to reduce mortality by 50% in high-dose *Vibrio*-inoculated iron-loaded mice in

contrast to a survival rate of 40% in DX p.o. and CIP treatment groups. In CIP plus CTR and DX p.o. plus CTR-treated groups, all mice died within 36 hr and 20 hr, respectively. Indeed, all combination groups were highly significant when compared with the control group. In contrast, only CTR combined with DX i.p. ($P = 0.01$) seems to be effective versus CTR combined with DX p.o. in the present mouse model of infection.

The qPCR results of both groups, with or without ethidium bromide monoazide (EMA) treatment, were not statistically significant ($P > 0.05$). Among the different antibiotic-treated groups, the number of DNA copies in DX p.o. and CTR-injected mice at 3 to 6 hr were not significantly different from the DNA copies of control mice ($P > 0.05$). However, the number of DNA copies in CIP-, TG-, and DX i.p.-treated mice were lowered by 1 $\log_{10}$ copies/µL as compared to the control groups (1×10^5 copies/µL), even in 3 to 6 hr. There was no significant difference in DNA copies among nonsurviving mice in across any of the combination groups. Despite this, the bacterial loads at 9 hr and 12 hr in CIP, DX i.p., and TG monotherapy-treated mice were reduced by 2 $\log_{10}$ copies/µL in contrast to combination therapy.

Bacterial load might not be the single cause of death in mice at early hours. The conclusion drawn by Hor *et al* [63] might be applicable to this research. *V. vulnificus* bacteria stimulated the production of TNF-alpha and blocked the migration of neutrophils. However, circulating bacterial DNA copies in surviving mice at 12 hr and 24 hr were significantly reduced, which could be helpful toward understanding the general principal of DNA load and mortality in mice. Indeed, the number of DNA copies is reduced in accordance with the increase in survival time of the mice. The numbers of DNA copies in

surviving mice at 24 hr in different treatment groups ranged from 1×10^1 to 1×10^2 copies/μL, which could be a useful marker for predicting the chances of survival after antibiotic therapy.

In summary, bacterial loads in *V. vulnificus*-infected patients can be quantified using qPCR, and DNA load can be used as a marker of disease severity and ultimately as a prognostic indicator of a patient's condition. Guidelines for selecting an aggressive treatment plan to reduce patient mortality may be available in the near future.

5.　　CONCLUSIONS

V. vulnificus-susceptible patients receiving regular blood or RBC transfusions or with thalassemia might be treated with DFS or DFP instead of DFO. DFS, a newly developed iron-chelating drug, potentially has superior clinical applications in iron-chelation therapy to prevent *Vibrio* sepsis by reducing iron availability.

In an unavailable condition of intravenous DX, CIP alone might be preferred option for reducing the mortality of *V. vulnificus* infected patients. However combination therapy of CTR plus DX i.v. would be the drug of choice for patients suspected with V. vulnificus septicaemia.

DNA load, quantified with qPCR, can be used for the identification of bacterial loads in *V. vulnificus*-infected patients. The DNA load subsequently can be used as a marker of disease severity to guide clinicians in prescribing aggressive treatment plans to reduce patient mortality.

REFERENCES

1. Bross, M.H., et al., *Vibrio vulnificus infection: diagnosis and treatment.* Am Fam Physician, 2007. **76**(4): p. 539-44.

2. Neupane, G.P. and D.M. Kim, *Comparison of the effects of deferasirox, deferiprone, and deferoxamine on the growth and virulence of Vibrio vulnificus.* Transfusion, 2009.49;1762-1769.

3. Stivers, T.L., *Diagnosing and treating deadly Vibrio vulnificus infection.* J Emerg Nurs, 2008. **34**(2): p. 139-41.

4. Tang, H.J., et al., *In vitro and in vivo activities of newer fluoroquinolones against Vibrio vulnificus.* Antimicrob Agents Chemother, 2002. **46**(11): p. 3580-4.

5. Starks, A.M., et al., *Pathogenesis of infection by clinical and environmental strains of Vibrio vulnificus in iron-dextran-treated mice.* Infect Immun, 2000. **68**(10): p. 5785-93.

6. Kikawa, K., et al., *A successfully treated case of Vibrio vulnificus septicemia with shock.* Jpn J Med, 1990. **29**(3): p. 313-9.

7. Chiang, S.R. and Y.C. Chuang, *Vibrio vulnificus infection: clinical manifestations, pathogenesis, and antimicrobial therapy.* J Microbiol Immunol Infect, 2003. **36**(2): p. 81-8.

8. CDC, *Vibrio vulnificus infections associated with raw oyster consumption-Florida, 1981-1992.* 1993.

9. Neupane, G.P. and D.M. Kim, *In vitro time-kill activities of ciprofloxacin alone and in combination with the iron chelator deferasirox against Vibrio vulnificus.* Eur J Clin Microbiol Infect Dis, 2010. **29**(4): p. 407-10.

10. Baethge BA, W.B., *Vibrio vulnificus: did Hippocrates describe a fatal case?* Rev Infect Dis., 1988 **10**(3): p. 614-5.

11. Roland, F.P., *Leg gangrene and endotoxin shock due to vibrio*

parahaemolyticus--an infection acquired in New England coastal waters. N Engl J Med, 1970. **282**(23): p. 1306.

12. Farmer, J.J., 3rd, *Vibrio ("Beneckea") vulnificus, the bacterium associated with sepsis, septicaemia, and the sea.* Lancet, 1979. **2**(8148): p. 903.

13. Hollis, D.G., et al., *Halophilic Vibrio species isolated from blood cultures.* J Clin Microbiol, 1976. **3**(4): p. 425-31.

14. Blake, P.A., et al., *Disease caused by a marine Vibrio. Clinical characteristics and epidemiology.* N Engl J Med, 1979. **300**(1): p. 1-5.

15. Matsuo, T., et al., *Fulminating lactose-positive Vibrio septicemia.* Acta Pathol Jpn, 1978. **28**(6): p. 937-48.

16. Goo JS, K.D., Han KS, Suk JS, Park MH, Kim SI. , *Lactose fermenting Vibrio (Vibrio vulnificus) septicemia: Report of five cases* Kor J Pathol, 1982. **16**:: p. 463-469.

17. Kim, J.J., et al., *Vibrio vulnificus septicemia: report of four cases.* Yonsei Med J, 1986. **27**(4): p. 307-13.

18. Yuan, C.Y., et al., *[Septicemia and gangrenous change of the legs caused by marine vibrio, V. vulnificus--report of a case].* Taiwan Yi Xue Hui Za Zhi, 1987. **86**(4): p. 448-51.

19. Powell, J.L., et al., *Release of tumor necrosis factor alpha in response to Vibrio vulnificus capsular polysaccharide in in vivo and in vitro models.* Infect Immun, 1997. **65**(9): p. 3713-8.

20. Kang, M.K., et al., *Induction of nitric oxide synthase expression by Vibrio vulnificus cytolysin.* Biochem Biophys Res Commun, 2002. **290**(3): p. 1090-5.

21. Linkous, D.A. and J.D. Oliver, *Pathogenesis of Vibrio vulnificus.* FEMS Microbiol Lett, 1999. **174**(2): p. 207-14.

22. Strom, M.S. and R.N. Paranjpye, *Epidemiology and pathogenesis of Vibrio vulnificus.* Microbes Infect, 2000. **2**(2): p. 177-88.

23. Lankford, C., *Bacterial assimilation of iron.* CRC Critical Reviews in

Microbiology 1973. **2**: p. 273-331.

24. Robins-Browne, R.M. and J.K. Prpic, *Effects of iron and desferrioxamine on infections with Yersinia enterocolitica.* Infect Immun, 1985. **47**(3): p. 774-9.

25. Bullen, J.J., et al., *Iron and infection: the heart of the matter.* FEMS Immunol Med Microbiol, 2005. **43**(3): p. 325-30.

26. Finkelstein, R.A., C.V. Sciortino, and M.A. McIntosh, *Role of iron in microbe-host interactions.* Rev Infect Dis, 1983. **5 Suppl 4**: p. S759-77.

27. Kushner, J., *Hypochronic anemias*, ed. C.t.o. medicine. Vol. 18 ed. Vol 1. . 1988, Philadelphia: WB Saunders, 892-900.: In: Wyngaarden JB, Smith LH, eds.

28. Kim, C.M., et al., *Ferrophilic characteristics of Vibrio vulnificus and potential usefulness of iron chelation therapy.* J Infect Dis, 2007. **195**(1): p. 90-8.

29. Choudhry, V.P. and R. Naithani, *Current status of iron overload and chelation with deferasirox.* Indian J Pediatr, 2007. **74**(8): p. 759-64.

30. Hor, L.I., T.T. Chang, and S.T. Wang, *Survival of Vibrio vulnificus in whole blood from patients with chronic liver diseases: association with phagocytosis by neutrophils and serum ferritin levels.* J Infect Dis, 1999. **179**(1): p. 275-8.

31. Simpson, L.M. and J.D. Oliver, *Siderophore production by Vibrio vulnificus.* Infect Immun, 1983. **41**(2): p. 644-9.

32. Wright, A.C., L.M. Simpson, and J.D. Oliver, *Role of iron in the pathogenesis of Vibrio vulnificus infections.* Infect Immun, 1981. **34**(2): p. 503-7.

33. Wright, A.C., et al., *Phenotypic evaluation of acapsular transposon mutants of Vibrio vulnificus.* Infect Immun, 1990. **58**(6): p. 1769-73.

34. Kim, C.M., et al., *Vibrio vulnificus vulnibactin, but not metalloprotease VvpE, is essentially required for iron-uptake from human holotransferrin.* Biol Pharm Bull, 2006. **29**(5): p. 911-8.

35. Kreger, A., L. DeChatelet, and P. Shirley, *Interaction of Vibrio vulnificus with human polymorphonuclear leukocytes: association of virulence with*

resistance to phagocytosis. J Infect Dis, 1981. **144**(3): p. 244-8.

36. Oliver, J.D., et al., *Production of extracellular enzymes and cytotoxicity by Vibrio vulnificus.* Diagn Microbiol Infect Dis, 1986. **5**(2): p. 99-111.

37. McPherson, V.L., et al., *Physiological effects of the lipopolysaccharide of Vibrio vulnificus on mice and rats.* Microbios, 1991. **67**(272-273): p. 141-9.

38. Johnson, D.E., et al., *Resistance of Vibrio vulnificus to serum bactericidal and opsonizing factors: relation to virulence in suckling mice and humans.* J Infect Dis, 1984. **150**(3): p. 413-8.

39. Park, N.Y., et al., *Identification of the Vibrio vulnificus wbpP gene and evaluation of its role in virulence.* Infect Immun, 2006. **74**(1): p. 721-8.

40. Park, N., Lee JH, Lee BC, Kim TS, and Choi SH, *Identification and characterization of the wbpO gene essential for lipopolysaccharide synthesis in Vibrio vulnificus.* J. Microbiol. Biotechnol. , 2006. **16**: p. 808-816.

41. Valiente, E., et al., *Vibrio vulnificus biotype 2 serovar E gne but not galE is essential for lipopolysaccharide biosynthesis and virulence.* Infect Immun, 2008. **76**(4): p. 1628-38.

42. Cappellini, M.D., et al., *A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with beta-thalassemia.* Blood, 2006. **107**(9): p. 3455-62.

43. Cappellini, M.D., *Exjade(R) (deferasirox, ICL670) in the treatment of chronic iron overload associated with blood transfusion.* Ther Clin Risk Manag, 2007. **3**(2): p. 291-9.

44. Olivieri, N.F., et al., *Iron-chelation therapy with oral deferipronein patients with thalassemia major.* N Engl J Med, 1995. **332**(14): p. 918-22.

45. Porter, J.B., *Practical management of iron overload.* Br J Haematol, 2001. **115**(2): p. 239-52.

46. Bowdre, J.H., J.H. Hull, and D.M. Cocchetto, *Antibiotic efficacy against Vibrio vulnificus in the mouse: superiority of tetracycline.* J Pharmacol Exp Ther,

1983. **225**(3): p. 595-8.

47. Perry, R.D. and R.R. Brubaker, *Accumulation of iron by yersiniae.* J Bacteriol, 1979. **137**(3): p. 1290-8.

48. Carniel, E., D. Mazigh, and H.H. Mollaret, *Expression of iron-regulated proteins in Yersinia species and their relation to virulence.* Infect Immun, 1987. **55**(1): p. 277-80.

49. (CLSI), C.a.L.S.I., *Performance Standards for Antimicrobial Susceptibility Testing; Eighteenth Informational Supplement.* 2008 Wayne (PA): The committee, .

50. Kim, D.M., et al., *In vitro efficacy of the combination of ciprofloxacin and cefotaxime against Vibrio vulnificus.* Antimicrob Agents Chemother, 2005. **49**(8): p. 3489-91.

51. Chuang, Y.C., et al., *Minocycline and cefotaxime in the treatment of experimental murine Vibrio vulnificus infection.* Antimicrob Agents Chemother, 1998. **42**(6): p. 1319-22.

52. Kim, D.M., et al., *In vitro efficacy of the combination of ciprofloxacin and cefotaxime against nalidixic acid-resistant Salmonella enterica serotype Typhi.* Int J Antimicrob Agents, 2010.

53. Drusano, G.L., et al., *Impact of short-course quinolone therapy on susceptible and resistant populations of Staphylococcus aureus.* J Infect Dis, 2009. **199**(2): p. 219-26.

54. Vidaillac, C., et al., *In vitro activity of ceftaroline alone and in combination against clinical isolates of resistant gram-negative pathogens, including beta-lactamase-producing Enterobacteriaceae and Pseudomonas aeruginosa.* Antimicrob Agents Chemother, 2009. **53**(6): p. 2360-6.

55. Kim, D., Yun NR, Chung JH *Time kill Studies of Antibiotics against a nalidixic Acid Resistant Salmonella enterica serotype Typhi.* Infect Chemotherapy 2008. **40:** : p. 207-211.

56. Moland, E.S., et al., *In vitro activity of tigecycline against multidrug-resistant Acinetobacter baumannii and selection of tigecycline-amikacin synergy.* Antimicrob Agents Chemother, 2008. **52**(8): p. 2940-2.

57. Mukherjee, P.K., et al., *Combination treatment of invasive fungal infections.* Clin Microbiol Rev, 2005. **18**(1): p. 163-94.

58. Kim, H.S., et al., *Comparison of conventional, nested, and real-time PCR assays for rapid and accurate detection of Vibrio vulnificus.* J Clin Microbiol, 2008. **46**(9): p. 2992-8.

59. Truccolo, J., et al., *Quantitative PCR assay to evaluate ampicillin, ofloxacin, and doxycycline for treatment of experimental leptospirosis.* Antimicrob Agents Chemother, 2002. **46**(3): p. 848-53.

60. Lee, J.L. and R.E. Levin, *Discrimination of gamma-irradiated and nonirradiated Vibrio vulnificus by using real-time polymerase chain reaction.* J Appl Microbiol, 2008. **104**(3): p. 728-34.

61. Lee, J.L. and R.E. Levin, *Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction.* J Microbiol Methods, 2006. **67**(3): p. 456-62.

62. Wang, S. and R.E. Levin, *Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR.* J Microbiol Methods, 2006. **64**(1): p. 1-8.

63. Hor, L.I., et al., *Mechanism of high susceptibility of iron-overloaded mouse to Vibrio vulnificus infection.* Microbiol Immunol, 2000. **44**(11): p. 871-8.

64. Kim, C.M., Y.J. Park, and S.H. Shin, *A widespread deferoxamine-mediated iron-uptake system in Vibrio vulnificus.* J Infect Dis, 2007. **196**(10): p. 1537-45.

65. Lesic, B., J. Foulon, and E. Carniel, *Comparison of the effects of deferiprone versus deferoxamine on growth and virulence of Yersinia enterocolitica.* Antimicrob Agents Chemother, 2002. **46**(6): p. 1741-5.

66. Litwin, C.M., T.W. Rayback, and J. Skinner, *Role of catechol siderophore*

synthesis in Vibrio vulnificus virulence. Infect Immun, 1996. **64**(7): p. 2834-8.

67. Kontoghiorghes, G. and E. Weinberg, *Iron: mammalian defense systems, mechanisms of disease, and chelation therapy approaches.* Blood Rev, 1995. **9**(1): p. 33-45.

68. Kim, D.M., et al., *Deferasirox plus ciprofloxacin combination therapy after rapid diagnosis of Vibrio vulnificus sepsis using real-time polymerase chain reaction.* J Infect, 2008. **57**(6): p. 489-92.

69. Ibrahim, A.S., et al., *The iron chelator deferasirox protects mice from mucormycosis through iron starvation.* J Clin Invest, 2007. **117**(9): p. 2649-57.

70. Piga, A., et al., *Randomized phase II trial of deferasirox (Exjade, ICL670), a once-daily, orally-administered iron chelator, in comparison to deferoxamine in thalassemia patients with transfusional iron overload.* Haematologica, 2006. **91**(7): p. 873-80.

71. van Asbeck, B.S., et al., *Synergy between the iron chelator deferoxamine and the antimicrobial agents gentamicin, chloramphenicol, cefalothin, cefotiam and cefsulodin.* Eur J Clin Microbiol, 1983. **2**(5): p. 432-8.

72. Engelman, J.A., et al., *Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers.* Nat Med, 2008. **14**(12): p. 1351-6.

73. Chait, R., A. Craney, and R. Kishony, *Antibiotic interactions that select against resistance.* Nature, 2007. **446**(7136): p. 668-71.

74. Jawetz, E. and J.B. Gunnison, *Antibiotic synergism and antagonism; an assessment of the problem.* Pharmacol Rev, 1953. **5**(2): p. 175-92.

75. Jawetz, E., J.B. Gunnison, and R.S. Speck, *Antibiotic synergism and antagonism.* N Engl J Med, 1951. **245**(25): p. 966-8.

76. Rahal, J.J., Jr., *Antibiotic combinations: the clinical relevance of synergy and*

antagonism. Medicine (Baltimore), 1978. **57**(2): p. 179-95.

77. Al Dahouk, S., et al., *Failure of a short-term antibiotic therapy for human brucellosis using ciprofloxacin. A study on in vitro susceptibility of Brucella strains.* Chemotherapy, 2005. **51**(6): p. 352-6.

78. Brill, J.R., *Diagnosis and treatment of urethritis in men.* Am Fam Physician, 2010. **81**(7): p. 873-8.

79. Chuang, Y.C., et al., *In vitro synergism between cefotaxime and minocycline against Vibrio vulnificus.* Antimicrob Agents Chemother, 1997. **41**(10): p. 2214-7.

80. Shakil, S., M. Akram, and A.U. Khan, *Tigecycline: a critical update.* J Chemother, 2008. **20**(4): p. 411-9.

81. Entenza, J.M. and P. Moreillon, *Tigecycline in combination with other antimicrobials: a review of in vitro, animal and case report studies.* Int J Antimicrob Agents, 2009. **34**(1): p. 8 e1-9.

82. Liu, C.Y., et al., *In vitro activities of tigecycline against clinical isolates of Aeromonas, Vibrio, and Salmonella species in Taiwan.* Antimicrob Agents Chemother, 2008. **52**(7): p. 2677-9.

83. Breedt, J., et al., *Safety and efficacy of tigecycline in treatment of skin and skin structure infections: results of a double-blind phase 3 comparison study with vancomycin-aztreonam.* Antimicrob Agents Chemother, 2005. **49**(11): p. 4658-66.

84. Vouillamoz, J., et al., *In vitro activities of tigecycline combined with other antimicrobials against multiresistant gram-positive and gram-negative pathogens.* J Antimicrob Chemother, 2008. **61**(2): p. 371-4.

85. Gunnison, J.B., et al., *Studies on antibiotic synergism and antagonism: the effect in vitro of combinations of antibiotics on bacteria of varying resistance to single antibiotics.* J Bacteriol, 1953. **66**(2): p. 150-8.

Acknowledgements:

Foremost my utmost reverence and gratitude goes to **Professor, Dr. Dong-Min Kim** for giving me research ideas with great admiration and inspiration whenever necessary during my entire academic period. This thesis would not have been possible without his support and guidance. I would like to thank Professor, Dr. Sook-Jin Jang for her crucial suggestions during my research period, and staying as part of committee member. I'm also grateful to Professor, Dr. Cheol-Hee Choi, Professor, Dr. Sung-Chul Lim and Professor, Dr. Sung-Heui Shin for their willingness to serve on my thesis committee and for critical review of my dissertation research.

I'm thankful to the Research Center for Resistant Cells of Medical Research Center (MRC), Chosun University and Korean Government for providing me the Korea Research Fellowship during my study period. It's pleasure to pay tribute to everyone in my laboratory for creating a great ambience for working and their invaluable assistance. I would really like to thank my colleague Dr. Sujan Piya and Dr. Jaya Ram Simkhada for their valuable companionship in each and every moment.

It's pleasure to express my gratitude wholeheartedly to my parents Shiva Prasad Neupane and Tulasha Neupane for imparting the right attitude to achieve my goals. My father, you always gave me the moral support and created study environment from my childhood. My mother is the person who sincerely raised me with her caring and gently loves. My siblings Sita, Shankar, Sharada and Punam, brother in law Mr. Narayan Sapkota and Sangita didi and all relatives; all of you are equally grateful for providing me your love and affection in my present position.

Last but not the least, my deepest gratitude goes to my sweetheart Kalpana whose dedication, love and persistent confidence in me. My dear, without your backing this would have been impossible. I gratefully acknowledge my both sons Aayush and Aavash for their continuous love and understanding throughout my study period. My dear kids, I'm sorry for always being very far from both of you in your early childhood period.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the study.

Ganesh Prasad Neupane

August 10, 2010

저작물 이용 허락서

학 과	의학과	학 번	20067757	과 정	박사
성 명	한글:네요파네 가네스 퍼러사더 영문: Ganesh Prasad Neupane		한문 :		
주 소	광주광역시서석동 467-4				
연락처	E-MAIL: neupaneganesh@yahoo.com				
논문제목	한글 : 비브리오 패혈증의 효과적인 치료약제에 대한 평가 영어: Evaluation of chemotherapeutic combinations against *Vibrio vulnificus* infection				

　　본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가
저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다　　　음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의
복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만,
저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사
표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을
경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에
의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한
저작물의 전송·출력을 허락함.

동의여부 : 동의(o　) 　반대(　　)

2010 년　 08 월　 25 일

저작자: Ganesh Prasad Neupane (서명 또는 인)

조선대학교 총장 귀하