IDENTIFICATION OF DIFFERENCES IN VIRULENCE FACTORS PRODUCTION FROM UV IRRADIATED ISOLATE OF CLINICAL *Vibrio cholerae* S

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Abstract

Mutants from the clinical isolate of *Vibrio cholerae* S were isolated after UV irradiation on the basis of antibiotic resistance for Rifampcin, Streptomycin and Klindamycin. Results revealed that the highest antibiotic resistant incidence is for Rifampicin after UV exposure for 15 minute.

One thousand mutant isolates were examined and showed smaller sized colonies on TSA appeared as smooth and translucence with yellow color on TCBS agar and pale on MacConkey agar. The mutant isolates gave positive results with oxidase and string test. Autoagglutination range from high level order designated +++ to mild ++ and low level designated as +. Only 15% of Rifampicin resistant mutant isolates gave no agglutination phenomenon. UV treated isolates produced delayed type of proteases with no changes in haemolycin and lipases production while, slightly autoagglutination has been detected from these mutant. Twenty out of screened isolates produced 10-30U/ml of CT at non- permissive conditions.

The toxin extract from mutant isolates symptoms such as muscle spasm, muscle cramp, exhaustion, trummer and tachycardia in mice after intraperitoneal injection. Erythemal effect and induration in the injected site of Guinea pig skin after intradermal injection remained even after more than one week, with pronounced bleeding detected in the injected area.

تشخيص الاختلافات في انتاج عوامل الضراوة من عزلة مشعة بالأشعة فوق البنفسجية للبكتريا المرضية *Vibrio cholerae* S

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الخلاصة

تم عزل طافرات من العزلة المرضية *Vibrio cholerae* S اعتمادا على المقاومة للمضادات الحيوية Rifampicin وStreptomycin وKlindamycin بعد التشعيع بالاشعة فوق البنفسجية وقد اثبتت النتائج ان تكرار المقاومة للمضاد الحيوي Rifampicin هو الاعلى بعد التعريض للاشعة فوق البنفسجية لمدة ١٥ دقيقة.

فحصت الف عزلة طافرة وقد اظهرت مستعمرات صغيرة الحجم على وسط TSA وظهرت كمستعمرات شفافة غير مجعدة ذات لون اصفر على وسط TCBS ولون شاحب على وسط MacConkey. اعطت المستعمرات الطافرة نتائج موجبة لفحص الاوكسيديز وفحص تكوين الخيوط. تراوحت ظاهرة التكتل الذاتي للبكتريا بين الشديد +++ والمتوسط ++ والقليل + وكانت ١٥% من العزلات المقاومة للريفامبسين خالية من ظاهرة التكتل الذاتي. اعطت العزلات المعاملة بالاشعة فوق البنفسجية انتاج متأخر من البروتييز مع انتاج طبيعي من الانزيم الحال للدم والانزيم الحال للدهون بينما لوحظ كمية قليلة لظاهرة التكتل الذاتي. تم التحري عن انتاج ذيفان الكوليرا في عشرين عزلة طافرة وقد تراوح الانتاج بين ١٠-٣٠ وحدة/ مليلتر في ظروف غير انتاجية.

سبب حقن مستخلص ذيفان الخلايا الطافرة في بريتون الفأران اعراض مثل التقلصات وتشنجات عضلية واعياء وتسارع دقات القلب. ولوحظ احمرار وتقلص في جلد خنازير غينيا استمر لأكثر من اسبوع مع نزيف ملاحظ في الاماكن المحقونة.

Introduction

The study of any microorganisms properties can be greatly enhanced by the generation of mutation in genes of interest. Creation of mutations and subsequent genetic mapping can elucidate the identity, relative size, number and organization of genes involved in a physiological process. Also, the recognition and location of transcriptional units can be defined by mutation and mutagenesis is used to create strains with desired proprieties, such as the ability to overproduce a desired metabolite or enzymes. Mutation of a gene or genes under study can be first altering the DNA of micro-organisms which determines the information and characteristics of cell; however the mutation is rare in nature. It cause a problem of an investigator is trying to a mass collection of a specific type of a mutation for genetic study or microorganism improvement. To increase the mutation frequency three general treatment can be used to mutagenized microorganism: electro-magnetic radiation, chemical mutagens, and transposons.

Mutation by electromagnetic radiation involves X-ray and gamma-ray , fast neutron and the more popular use the ultraviolete light [1]. Germicidal range of UV which effectively inactivate bacteria and viruses could be used between 200-280 nm [2]. Miller [3] mentioned that UV induces a wide spectrum of mutation in *Escherichia coli*, it causes base substitution, and an effective deletion mutation, in which DNA absorbing the UV casing cross linking between neighboring pyrimedene nucleoside bases in the same DNA strand. Due to the mutated base, formation of the hydrogen bonds to the purine bases on opposite strand is impaired, leading to block of DNA transcription and replication causing the cell death. Also, Oguma [4] mentioned that UV B and C(220-320 nm) radiation effected through the formation of lesions the genomic DNA. The presence of UV induced lesions would inhibit the normal replication of DNA and therefore result in inactivation of the microorganism. However, DNA repair mechanisms are take place so light and dark mechanism are used by some bacteria to repair UV-induced damage. Exposure to UV light for a short time results in the induction prophage such as lambda and Ø80 [3, 5]. Faroque *et al* [6] found that the exposure of cultures of CTXØ lysogenes to direct sunlight resulted in 1000 fold increase in phage titer. The effect of sunlight was roughly proportional to the product of light intensity and duration of exposure. Davis [7] explained the induction of a prophage CTX Ø of *V. cholerae* that CTX Ø have copies of RS1 a satellite phage whose transcript depends upon properties produced from a CTX prophage its helper phage. However, RS1 can aid the CTX as well as exploit it due to RS1-encoded protein RSTC which acts as antirepressor that counteract the activity of phage repressor, RSTR. RSTC production resulted in increased transcription level for *ctx*AB at non- permissive conditions. Faroque *et al* [8] subjected *V. cholerae* strains to UV irradiation to induce possible prophage carried out by strains. Also, Al- Khafaji [9] found a stimulation of Toxin Coregulated Pili (TCP) and Cholera toxin (CT) production at non- permissive conditions after short time of UV exposure.

Faroque *et al* [10] explained the production of CT at non- permissive conditions in carrying mutations in either toxR, toxS, tcpP, or tcpH.

On the other hand, the use of UV in mutation experiments are easy to maintain and need no additional chemical input. More over, UV irradiation produces no hazardous by products comparing with many of the conventional chemicals.

This research came to study the effects of UV irradiation on *V. cholerae* S isolate, the capability of mutant isolates to produce CT at non- permissive conditions.

Materials and Methods

Thiosulphate Citrate Bile Salt (TCBS) from Amersham and tryptone Soy agar (TSA) and Blood agar Base (BAB) from Mast Diagnosis media were prepared as recommended by the manufacture while AKI (peptone 1.5, yeast extract 0.5 and NaCl 0.4 pH 6.9), production media (AKI plus 0.2% glucose and 0.2% asparagines pH 8.5) and Lauria Birtani broth (LB) (Tryptone 1%, Yeast extract 0.5% and Sodium chloride 0.5%) were prepared and sterilized by autoclaving at 1.5 bar for 15 min [5, 9, 11].

Sample collection and isolation of *V. cholerae*

Fifteen clinical isolates were collected from diarrheal stool samples in Central Health Laboratory, Baghdad, Ministry of Health. The characterization was confirmed by performing biochemical tests, and serotyping according to Baumann and Schubert [12]; Elliot *et al*. [13]; and WHO [14].

Detection of TCP

Vibrio cholerae hydrophobicity increased in broth culture due to the expression of pili causing visible clumping of bacteria as a pellet at the bottom of tube and leaving a clear supernatant this phenomenon is known as autoagglutination which can be recognized by naked eye [9, 15].

Extraction of CT

The steps for the detection of CT depend on extraction by centrifugation at 5000 rpm for 10min, concentration using 80% saturation of Ammonium Sulfate, and desalting of toxin protein through gel filtration by Sephadex G25 before applying to the animal models [16, 17].

Detection Methods of CT

Qualitative detection of CT was done using two animal models for *in vivo* screening of toxins, the mouse lethality assay after intraperitoneal injection of toxin extract and the Guinea pigs permeability factor (PF) after intradermal injection of toxin extract [18, 19].

Toxin Units was calculated (depends on its definition) for quantitative measurement in which each 5-8 mm of EA is equivalent to 1 Toxin Unit (TU) of enterotoxin.

$TU/ml = (EA\ mm \div 5) \times 10$

The isolate designated *V. cholerae* S was chosen for production of cholera toxin after mutagenesis at non- permissive conditions because its higher production of CT among the other isolates.

Specific toxin activity was calculated as the ratio of TU/ml divided by the protein concentration [18, 19].

Antibiotic susceptibility

V. cholerae S isolate tested for its susceptibility to Rifampicin, Streptomycin and Klindamycin by standard disc agar diffusion method [20] and again by antibiotic incorporating agar as described by Sambrook *et al*. [5] with antibiotic reached to 100µg/ml for Rifampicin and streptomycin and 250µg/ ml for Klindamycin following Miller [3].

UV irradiation

UV was used to mutate *V. cholerae* S by a new method as follows:

Ten ml of overnight culture of *V. cholerae* S was washed with phosphate buffer 0.15 M pH 5.5 then bacterial pellet was suspended with the same buffer.

0.1ml of each sample was removed and serial dilution were made for colony counting of zero time.

Bacterial suspension was placed on sterilized quartiz cuvate and UV irradiation was exposed from spectrophotometer equilibrated at 260 nm.

Serial dilutions were made after UV irradiation exposure from 5- 30 minute with 5 minute interval and olated over BAB.

Only 1ml of each treatment was used to inoculate 10ml of LB broth and incubated for 18 hours at 37ºC shaker incubator. Overnight cultures were screened for autoagglutination while, 0.1ml was streaked over BAB with Streptomycin or Rifampicin or Klindamycin.

Production of virulence factors from mutant isolates

One thousand mutant isolates were screened for their differences in virulence factors production, morphological characters and autoagglutination at non-permissive conditions (AKI pH6.9 and 37ºC).

Morphological character

Mutant isolates of *V. cholerae* S was streaked over the TCBS agar, MacConkey agar and TSA, all incubated overnight at 37ºC. Colony shape and their appearance on each agar was compared with wild *V. cholerae* S.

The production of Proteases, Lipases, Haemolysins

Only 5 µl of each bacterial growth from antibiotic resistant mutants were spotted on TSA containing either 1% casein or 1% tween 80 or 7% sheep blood and incubated for 18-48 hour at 37°C. Cleared or turbid zone was used to detect proteases and lipases respectively [9]. The type haemolysis on blood agar determined the production and type of haemolysin [13].

Autoagglutination Phenomenon

AKI broth pH6.9 was inoculated with the mutant isolates and incubated at 37ºC for 18 hour in 110 rpm shaker incubator, autoagglutination was screened and compared with wild isolate of *V.cholerae* S cultured at the same conditions.

CT production

Twenty out of one thousand mutant isolates were chosen and cultured at AKI broth pH 6.9 while, the wild isolate *V.cholerae* S was cultured at both AKI broth pH 6.9 and producing medium. Bacterial free extract was used to measure the EA and the amount of CT production.

The biological activity of CT

Crude preparation of the mutant and wild type of CT was used to determine: Mice lethality of toxin to white mice in which two animal of Balb/c mice were injected intraperitonially (I. p) with 0.1 ml of toxin extracts then mice were watched for 24 hour for the appearance of symptoms or death [9, 18].

Also Erythemal activity of Guinea pig was studied in which the animal was injected with 0.1 ml of crude extract of CT intradermally (I. d) in the shaved dorsal area after dividing it into 12-18 equal square. Erythema and induration area resulted from injected toxin extract was measured and the animal was kept under observation for 72 hour. [18, 19].

Results and Discussions

The mutation of *V. cholerae* S

The rarity of mutations is a problem if an investigator is trying to a mass collection of a specific type of mutation to serve study purpose. Researchers respond to this problem in two ways, one of them is to increase the mutation rate using mutagenic agents that have the biological effect of inducing mutations above the background, or spontaneous rate.

In recent investigation UV irradiation was used in mutation and antibiotic resistant for Rifampicin, Streptomycin and Klindamycin were screened as mutation indicators.

Coleman *et al*. [21] explained that these three genetic determinants responsible for the antibiotic resistant could consider as a stable genetic markers because they reside on bacterial chromosome comparing with other antibiotic determinants harbor on mobile genetics element which may lost during bacterial routine subculturing or at elevated temperatures.

Agitated LB used for preparing bacterial culture in which *V. cholerae* S grew as homogenized culture facilitating the equal distribution of UV irradiated. Also, agitated LB gave homogenized culture with high bacterial number 10^8 cfu/ ml after 12 hour incubation at 37°C. These criteria serve the experimental purpose as jugged by OECD [22] when recommended the use of late exponential or early stationary phase of bacterial growth which contained a high titer of viable bacteria. Also many workers recommended the use of LB in genetics experiments in order to avoid bacterial clumping and aggregation which may appeared when other types of broth were used [5].

The first step in employing the mutation experiments was checking the susceptibility of *V. cholerae* S isolate to phosphate buffer with pH 5.5 which used in bacterial suspension, because *V. cholerae* is very sensitive to low pH and might loose its viability in such environment, however our results reflect that no changes in bacterial number occurred after 60 minute incubation time on phosphate buffer pH 5.5 at 37°C (Table 1).

Table 1: The viable count of *V. Cholerae* S isolate incubated for different period in phosphate buffer pH 5.5

Incubation period min	Bacterial number CFU/ml
Zero	2×10^8
١٥	1×10^8
٣٠	1×10^8
٦٠	8×10^7

Following Calam [1] and OECD [22] when recommended the use of appropriate solvent for UV treatment which should not be suspected to

exposure reactions with the wave length and should be compatible with the survival of the bacteria.

UV treatment adopted at this study based on the capability of short UV wavelength especially at 260nm to induce mutation through absorption of UV light by DNA molecules.

The results obtained at Present work came through new method in UV irradiation and present procedure provided the critical factors achieve microbial inactivation by UV exposure. The transmissivity of the product, the geometric configuration of the reactor, the power ,wave length, physical arrangement of UV source and the radiation path length in which all of these criteria recommended by US Food and Drug Administration [2].

Results showed that *V. cholerae* S isolate exposed to UV for 15 minute gave the highest mutant frequency for the three antibiotic determinants. Mutant frequency for Rifampicin resistant reached to 2000 cfu mutant cell/ml. While Streptomycin and Klindamycin resistant mutant induced at low frequency. Only 50 and 55 cfu mutant/ml respectively can be detected after 15 minute of UV exposure as presented in Table 2.

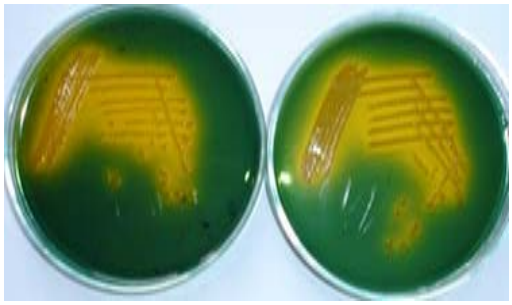
Table 2: The antibiotic resistant mutant colony after UV irradiation of *V.cholerae* S

UV exposure (min)	Rifampicin resistant (CfU mutant /ml)	Streptomycin resistant (CfU mutant /ml)	Klindamycin resistant (CfU mutant /ml)
5	20	-	-
10	80	-	-
15	2000	50	55
20	100	10	45
25	35	-	-
30	20	-	-

- = no mutant colony

Our results indicated that Rifampicin resistant is the most frequent among other used indicators and this may due to the large number of genes that responsible for RNA polymerase synthesis which consist of many subunits overlap between them. Also Rfampicin antibiotic binds to B´ subunit leading to cell death while any alter in the structure of one of these subunits after mutagen treatment led to loss the capability of Rifampicin in binding and killing effect causing cell resistant [23]. Our results were in agreement with Calam [1] when gave the range of UV exposure from ½ min – 20min depending on the sensitivity of the organism. Also Oguma [4] altered the dose of UV used for *Escherichia coli* disinfection by controlling the exposure time to UV irradiation. The low mutant frequency at longer time of UV exposure may due to the high killing event happened by UV or elevated suspension heating at the reactor resulting in accidental death of cells from a cause other than the primarily used for mutation.

All screened UV light mutant colonies of *V.cholerae* S isolate showed smaller sized colonies (1-2 mm diameter) on TSA medium comparing with wild type colonies. Each mutant colony appeared as smooth and translucence with yellow color on TCBS agar (Figure 1) and pale on MacConkey agar plate. Also the mutant isolates gave positive results with oxidase and string test.



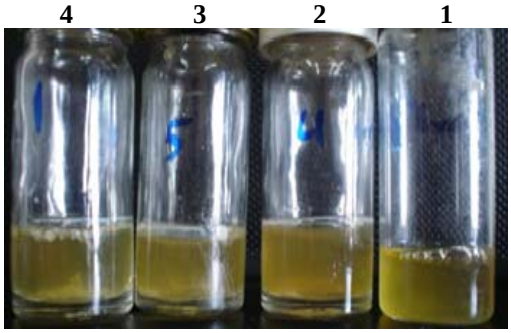
A **B**

Figure 1: Growth of wild type (A) and mutant isolate (B) of *V. cholerae* S on TCBS agar

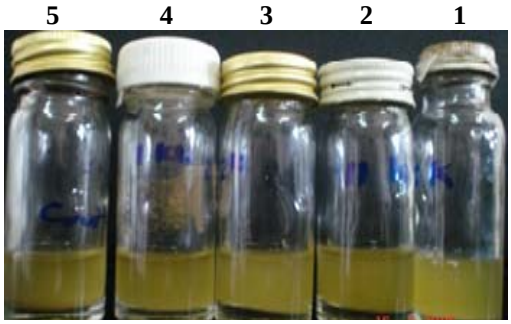
The mechanism in which UV light caused either mutation or cell death described by OECD guideline [22]; US Food and Drug Administration [2]. DNA absorbing the UV light caused cross linking between neighboring pyrimidine nucleoside bases (thymine and cytosine) in the same DNA strand. Due to the mutant bases formation of the hydrogen bonds to the purine bases on the opposite strand impaired so DNA transcription and replication is blocked comprising cellular function and eventually leading to cell death. However, as a result of exposure to UV irradiation from sunlight, many organisms, have developed mechanisms to compensate for the damaging effects of UV irradiation. These organisms can possess multiple pathways to repair UV induced DNA damage as nucleotide excision repair and photoreactivation repair which both discussed by Thoma [24]. On the other hand, Zimmer and Slawson [25] found that *E. coli* underwent photorepair following exposure to low pressure UV source, but no repair was detectable following exposure to medium- pressure UV lamp. Cell death or level of mutation event depends proportional to the amount of UV

exposure and UV repair system present in the target microorganism.

For further characterization 1000 mutant isolates were taken from different treatments in which proteases, lipases and haemolysin assayed and compared with the production of wild type isolate of *V. cholerae* S. Also, autoagglutination screened at non permissive conditions qualitatively and the comparison made with wild type culture at the same experimental condition. Results showed that autoagglutination range from high level order designated as +++ to mild ++ and low level designated as + (Figure 2). However, 15% of Rifampicin resistant mutant isolates gave no agglutination phenomenon as shown Figure 2B.



A- LB broth for *V.cholerae* S (1) and the high level of autoagglutination mutant isolates (2, 3, 4).



B- LB broth for *V.cholerae* S(1) and the low level of autoagglutination mutant isolates (2,3,4,5).

Figure 2: Autoagglutination phenomenon in LB broth for *V. cholerae* S and mutant isolates

Mutant isolates of *V.cholerae* S screened after UV treatment produced delayed type of proteases with no changes in haemolysin and lipases production (Figure 3) while, slightly autoagglutination could be detected from these mutants.

The delayed type of proteases production may due to the secretion pathway of proteases which may affected by mutation in which secreted protein required for extracelluar transfer of proteases were mutated and dysfuncted.

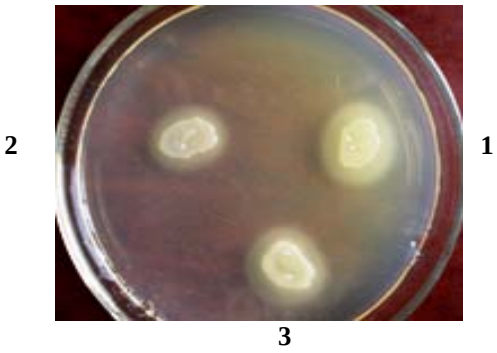


Figure 3: Lipases produced by wild type of *V. cholerae* S (1) and the mutant isolates (2, 3).

The delayed type of proteases production may due to the secretion pathway of proteases which may affected by mutation in which secreted protein required for extracelluar transfer of proteases were mutated and dysfuncted. On the other hand, type II secretion apparatus is composed of at least thirteen different proteins, and these components are localized to the cytoplasmic membrane lead to many alteration in genes encoded these proteins caused a defect in secretion pathway. Sandkvist *et al* [26] and Scott *et al* [27] characterized that Hap protein secreted to the culture medium through the general secretary pathway which encoded by the extracellular protein secretion genes.

The mutant isolates of *V. cholerae* S showed delayed type of proteases activity with low level of TCP production at non permissive conditions might have a defect at one or more of regulatory proteins. Zhu *et a.l* [28] demonstrated that the well characterized ToxR signal transduction cascade responsible for sensing and integrating the environmental information and controlling the virulence regulon. Dirita and Mekalanos [29] suggested that the expression of a subset of virulence factors of *V.cholerae* is coordinately controlled by a regulatory cascade. The mutant at *tox*R which gained by Bina *et al.* [30] showed the dramatic transcriptional changes compared with the wild type. A total of 154 genes showed 1/2 fold change in transcription, 60 genes showed increased expression in the mutant strain and 94 genes showed decreased expression in the mutant strain. Many of the *tox*R activated genes involved in the production of TCP and CT. On the other hand, quorum sensing regulators are involved in regulation of *V. cholerae* virulence genes in response to cell density as Kovacikovra and Skorupski [31] found that the mutation in delta *hap*R gene (the positive regulator of HA protease) caused

increase expression of a *ph*A gene responsible in activation of *tcp* and *ctx* gene. At the same respect, Vance *et al.* [32] confirmed the role of LuxO as a central "switch" that coordinately regulates virulence –related phenotype such as protease.

The appearance of low level of autoagg-lutination after mutation of *V. cholerae* S in this study may related with a defect in pilli biosynthesis pathway agreement with Kaufman [33] and Nesper *et al* [34] who characterized and found that the *gal*U and *gal*E mutants gene and rough-LPS mutant comprising altered core oligosaccharide.

Also, several genes from the ancestral genome have functions in the normal bacterial physiology and have been found to have global effect in the control expression of the virulence genes.

Twenty out of mutant isolates giving different quantity of autoagglutination phenomenon were chosen and screened for CT production at non-permissive conditions. All screened isolates produced CT at non-permissive conditions and gave 10-30 U/ml compared with no production of CT by wild type *V. cholerae* S isolate (Table 3).

Table 3: Comparison of Autoagglutination, proteases and CT production between UV mutant isolates and the wild type *V.cholerae* S

Bacterial isolate*	Resistant marker	Protease	Autoagg-lutination	EA (mm diameter)	Toxin unit/ml
UV1	Rifampicin	-	+++	15	30
UV2	Rifampicin	-	+++	12	24
UV3	Rifampicin	-	+++	15	30
UV4	Rifampicin	+	-	-	-
UV5	Rifampicin	+	-	-	-
UV6	Rifampicin	delayed	++	12	24
UV7	Rifampicin	-	+	5	10
UV8	Rifampicin	delayed	++	10	20
UV9	Rifampicin	delayed	+	7	14
UV10	Rifampicin	-	+	6	12
UV11	Rifampicin	+	-	-	-
UV12	Rifampicin	+	-	-	-
UV13	Rifampicin	+	-	-	24
UV14	Rifampicin	-	+++	12	24
UV15	Rifampicin	delayed	+++	12	24
UV16	Rifampicin10	-	++	10	20
UV17	Rifampicin	-	++	10	-
UV8	Klindamycin	delayed	-	-	20
UV19	Klindamycin	-	++	10	24
UV20	Klindamycin	+	-	-	
Wild type S**	-	+	++++	17	34
Wild type S*	-	+	-	-	-

-= negative
+=positive
***= AKI pH6.9 and 37º C**
****= production medium and 35 º C**

All extracted CT from screened mutant isolates gave biological activity seems to wild type CT biological activity. The extract from mutant isolates caused symptoms such as muscle spasm, muscle cramp, exhausted, trummer, and tachycardia were observed on mice after I.p injection. Erythemal effect and induration in the injected site of Guinea pig skin, remained even after more than one week, with pronounced bleeding detected in the injected area (Figure 4).

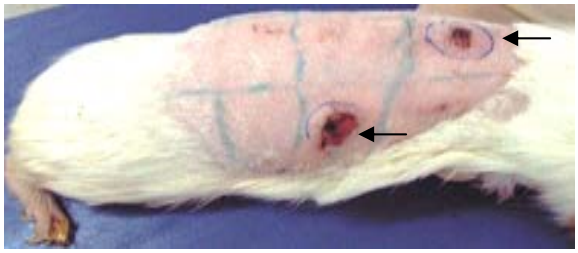


Figure 4: Permiability factor in Guinea pig skin I. d injected by CT (crude extract of mutant isolate1) top and (crude extract of wild type CT) below

Richardson [18] and Finklestein [35] mentioned that one of CT effects on various animal a characteristic delayed (maximum response at 24 hours), sustained (visible up to one week or more) erythematous, edematous induration asso-ciated with a localized alteration of vascular permeability responses appeared after intrad-ermal inoculation of relatively minute amount of CT reached to only 30 pg.

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