LABORATORY BOOK NO:_____ PROJECT CODE:_____

ISSUED TO: Prathicksha ON: 29/06/ 20 20

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FROM BOOK NO:_____

Good Laboratory Practice Notebooks

COMPLETED:_____ 20 _____ COPIED:_____ 20 ____

ORIGINAL STORED ROOM_____ COPY STORED ROOM_____

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Notes

- curcumin is light sensitive → wrapped in foil
- different lab coats for each part of lab
- density. concentration of curcumin

500mg/ml $\frac{500}{1} = \frac{20}{x}$

1 + 3 + 3

MSSA MRSA

bacteria from patient ↓ ↓

sensitive staph. aureus resistant staph. aureus

↓ ↓

control be sensiti

$C_1V_1 = C_2V_2$

concentration / volume

$C_1 = 500$ $C_2 = 20$

$V_1 = 1$

$500 \times 1 = 20 \times x$

$500 = 20x$

$x =$

3 from freezer ↳ ask what they were

phage → ~~orange~~ orange phage

Day 1 29/6/20 - MORNING SESSION

- made agar concentrations with TSA & TSB powders
- swabbed the MSSA & MRSA bacteria in bunk room
- ~~placed~~ placed plates in ~~the~~ incubation
- measured curcumin (20mg) using calibrator
- added 40mg of water using pipette
- foiled lid
- shook tube in centrifuger
- placed in refridgerator

AFTERNOON SESSION

- curcumin adjustment because it did not dissolve ∴ used with diff-liquid
- made agar plates by pouring solution into empty plates inserted
- ~~placed~~ bacteria onto agar plates
- three types of orange phage: 1/3, 2/3, 3/3 were placed on bacteria ;

0 -1 -2 -3 -4 -5 -6 -7

1 → 1 = ○○○○○○

2 → 2 = ○○○○○

3 → 3 = ○○○○○

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today
- cell
- curcumin & phage levels so that phage does not die

Morning

[Making the cell culture - side experiment to see if the curcumin kills the cell]
- new lab, new lab coat (tissue sampling room)

- make the base for the cell's growth on plate
- mixed liquids & added the cell into tube
- centrifuged tube w/ another tubed liquid on opposite side for balance
- cell was isolated in the base
- vacuum took up the liquid in the cell's solution to isolate cell
- Sealed the CD08 coated plates & ~~refrigerated~~ incubated for 20 mins
- complete media was mixed w/ cell

[Bacteria w/ different concentrations of curcumin in 3.33×10^8 orange phage (2/3) to determine the effectiveness of them together]

- curcumin was incubated bc. it was frozen after refrigerating
- bacteria was swabbed & mixed into saline
- McFarland scale was used for bacteria in saline to become 0.5 - too small, add more bacteria, too large, add more saline
- curcumin was diluted in phage except 1 was control without curcumin
- Each S.aureus bacteria (S1, S2, S3, R1-6) was diluted in the phage and curcumin
- 0.4mg requires 40 microlitres

Afternoon
- placed each bacteria w/ each curcumin concentration in incubator
- Set up the biofilm protocol using S.aureus in 96 well plate
- Outer wells had PBS (200µl) ~~2 plates~~
- One line of broth (150µl) - 2 plates
- 150µl of bacterial mix in wells for biofilm formation
- Wrapped in foil to prevent evaporation
- incubated for 48hrs at 37°C on a rotating plate set at 70rpm

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MORNING — counting dots — day 3

1hr Phage + bacteria:

3 microlitre convert to ml

1.0 7×10^8 — $\frac{18+25+20}{3}\times\frac{1000}{3}\times10^5$

0.8 4×10^8 — $\frac{12+10+14}{3}\times\frac{1000}{3}\times10^5$

0.4 6.22×10^8 — $\frac{20+21+15}{3}\times\frac{1000}{3}\times10^5$

0.2 5.67×10^8 — $\frac{16+13+22}{3}\times$ (1000/3 × 10^5)

control 6.78×10^8 — $\frac{23+15+23}{3}\times\frac{1000}{3}\times10^5$

$20.3\times10^5\times\frac{1000}{3}$

Same amount of phage plaque shows that phage still active

no difference, curcumin won't destroy ph.

4hr Phage + bacteria

1:0 6×10^7 — $\frac{21+16+17}{3}\times\frac{1000}{3}\times10^4$

0.8 3.89×10^7 — $\frac{14+9+12}{3}\times\frac{1000}{3}\times10^4$

0.4 5.33×10^7 — $\frac{15+18+15}{3}\times\frac{1000}{3}\times10^4$

0.2 4.56×10^7 — $\frac{14+14+13}{3}\times\frac{1000}{3}\times10^4$

Control 5×10^7 — $\frac{13+19+13}{3}\times\frac{1000}{3}\times10^4$

~~Tested~~ Curcumin is safe because compared to the control, the results are relatively close.

24hr phage will be tested to see results tomorrow

+ curc

5% 1.5% TSB Plates

bacteria + TSB 4ml in each

100ml microlitres

-1 -2 -3 -4 -5 -6 -7

curc: Ctl, 0.2, 0.4, 0.8, 1.0 — 20 microlitres

Ctl . . . .

Sample size: 3 { ooooooo, ooooooo, ooooooo

phage titres are very different because test was NOT accurate, because we are assuming that 1 phage attacks one plaque

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PROJECT No.:_____ From Page No.:_____ Day 3

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AFTERNOON - make samples of curc & bacteria (curc alone)
- review results from yesterday's test (curc & ph)

- bacteria in saline
- different method, quicker but more complex
- source of error

- each 'ingredient' (bacteria - 2μl, curcumin - 20μl, brom - 178μl) was placed in every bacteria
  - RI - just bacteria
  - 0.2
  - 0.4
  - 0.8
  - 1.0

↑ results
- contamination in TSB

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add treatment to biofilm

MORNING
- reviewing the curcumin alone/bacteria results from yesterday
↳ there was contamination in the broth

- Reviewing the biofilm results
↳ all plates survived biofilm except there was contamination in the broth for R6 & R2

24hr phage/s.aureus

1.0 3.33×10^8 pfu
$\frac{0+2+1}{3} \times \frac{1000}{3} \times 10^5$

0.8 6.67×10^8
$\frac{3+2+1}{3} \times \frac{1000}{3} \times 10^5$

0.4 1.67×10^8
$\frac{6+5+4}{3} \times \frac{1000}{3} \times 10^5$

0.2 1.44×10^8
$\frac{4+5+4}{3} \times \frac{1000}{3} \times 10^5$

ctl 2.22×10^5
$\frac{4+3+3}{3} \times \frac{1000}{3} \times 10^5$

24 hr Curc/Phage S.aureus

AFTERNOON → EVENING
- made the curcumin alone w/bacteria samples again

- treated the grown biofilms
↳ aspirate the wells to isolate the biofilm on the bottom (vacuum suction) } done on 2x
↳ add 180µl PBS to the biofilms
↳ Aspirate again, making sure that the biofilms do not get sucked out
↳ wash w/PBS
↳ Aspirate

- add curcumin alone to designated wells
- add phage " "
- add c & p " to designated plate wells
- add the broth to the bacteria alone controls
- added the only broth wells
- incubate 37°C on a rotating plate

Why washed twice?

After

RATIOS

	Curc.	0.2 µg/ml	0.4 µg/ml	0.8 µg/ml	1.0 µg/ml
Ph.					
C+P					
Bact. Br					
Br					

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MORNING

- Reviewing the cell culture from Tuesday
  - took the cell out of the incubator
  - aspirated the old media
  - washed w/ TSB 6 ml x 2 each
  - looked through light microscope, aspirated in middle
  - saw that the cells were quite far apart, not confluent
  - placed in incubator 37°C

AFTERNOON

- record & review curcumin alone/bacteria on microplate reader
- conducting the crystal violet assay of the biofilms
- aspirate the planktonic bacteria w/ vacuum suction
  - wash w/ TSB
  - aspirate
  - wash w/ TSB
  - aspirate
  - leave for 10 mins
  - add 180 µl crystal violet 0.1% in water to each well except TSB (inc. broth, bacteria) ← same as planktonic cells
  - let sit for 15 mins, room temp. to stain
  - aspirate
  - wash 3x w/ TSB (180 µl)
  - when taking out TSB, plates were rotated and then flipped onto sink
  - plates were put in 55°C oven for 15 mins

NOT TSB inc. broth, bac

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questions

- the machine that produced the agar, how long it took?
- TSA or TSB that was poured on the Petri dishes?
- when the curcumin didn't dissolve, what liquid agent was used?
- why was orange phage used?
- why 2/3 orange phage was chosen
- Phage Titre: -1, -2, -3 meaning - dilution
- how much curc/phage was dotted on the plates
- brief overview of the cell culture test
- was PBS used to keep the moisture in the plates during 48hrs?
- measurements
- biofilm: how much cur in each well?
  - phage
  - broth
- bacteria: how much cur in each well?
  - phage
  - broth

Chamber slides for live/death analysis
to see how many bacteria are alive and dead

auto clave to sterilise at 121°C
to guarantee all bacteria in it are killed for no contamination
15mins

DMSO - high conc. of curc

prevent evaporation

absorb
↑
high OD → more alive bacteria

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DAY 6

Objectives: treat the cell with curcumin + created biofilm CV + AB + planktonic bacteria

50x Ex Plus media → Ex Plus Basal Media + Ex Plus supplement

dishes out of incubation
vaccuum the media — wash with PBS x2
add the trypledia
place in incubator for 10 minutes
make full media

setting up the biofilm

curcumin - eg. stock 10mg/ml

now → 10µl in 10 mL of water

Incubating because it was in -20°C for one week (for 1 hr) in 37°C

- review the five bacteria on a Hawksley plate with dye
- placed cells in media in 4 plates of 96 wells

Afternoon

set up w/ biofilm

1. Crystal violet - biomass of bacteria
+
2. Alamar blue - metabolic - reaction of bacteria & growth (proliferation)

CV

- dilution of bacteria in saline to be 2.0 McF
- 150µl of PBS on outer wells except 2 for DMSO
- DMSO acted as the control for the curcumin's liquid agent
- plates were labelled
- 2 µl of bacteria in designated wells
- DMSO in designated wells - find quantity
- added broth in designated wells, designated quantities
- placed in incubator rotating plate 37°C

Chambers ++

AB

- dark plates because the dye that will be added is light sensitive

$C_1V_1 = C_2V_2$

$10 \times x = 0.2 \times 200$

- Same procedure as the CV

Planktonic bacteria

- bacteria in dilution 0.5 McF
- one line of DMSO on each plate for each bacteria
- refer to photos of labelled plates (13/07/20, 5:29pm)
- applied bacteria
  curcumin
  phage
  broth

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Day 1

Objectives:
- treat the cell w/ curcumin to see the curcs toxicity to the human cell
- review the planktonic bacteria against ptr curc from yesterday

- get the cell in cell media out of incubation ↗ get out from incubator
- aspirate the cell media
- wash w/ PBS
- aspirate
- add the curcumin in quantities as on photo after
- 4 plates, same elements, 2 for 24hrs, 2 for 48hrs
- add the Ex Plus Complete Media for the cell to survive in
- place in incubator ✓

- review planktonic bacteria
- see which conc of curcumin would work w/ each bac
- aim for the lowest concentration

R1
1 ✓ 0.8
2 killed 2 killed

R2
~~0.4~~
~~3 killed~~
all alive

R3
1 / 3 killed
0.8+0.4 / 2 killed
↑ no improvement

C
C+P
Original 3 killed, 0.2

0.2 ✓ 0.4
3 killed

0.2
1 / 3 killed
0.8 2 killed
0.4 1 killed
0.2 1 killed

R4
1, 0.8, 0.4, 0.2
all killed

R5 sens to curc.
all killed

R6 0.2
all killed
ex. 0.2, all alive

S1 - sens to curc
all killed

C
C+P
all killed 0.2
exc. 1 on 0.8

all killed'd

all killed

all killed

S2
none killed
ex. 1, 0.2

S3
1, 0.8 ✓ all killed
0.4, 0.2
0.4 ↑ 2 killed
1 partial

RS of S1
sensitive
↳ reduce curc. concentration

C
C+P
1 ↓ all killed
0.8 ↓ 2 K
0.4 ↓ 2 K
0.2 ↓ 1 partial

1, 0.8 all killed
0.4 2 killed
0.2 all killed

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DAY 8

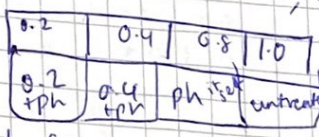
- treating the CV biofilms w/ curcumin & phage
- treating the AlamarBlue biofilms w/ curcumin & phage

Q.

phage titre formula
quantities of curc, phage
cell culture protocol

add to the figures → 2 DMSO dilution like how curc is diluted

Chambers are same, larger volume (350µl)



LDH ASSAY ← used for a different dye test

Cell Toxicity – Take out 2 plates (24hr plates) after

- ~~treated~~ x100 tritent 3 wells in untreated column 20µl
(acts as a control) positive → 100% will kill cell in each well
because it is used in LDH

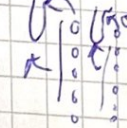
LDH is an enzyme that is released from a dead cell
high LDH released,

because we are comparing how toxic the curcumin is, we use Tritent X, to compare it with

negative control: untreated (cell media)

positive control: Tritent X 100% will kill cell

each group was collected in 2 test tubes



and the 3 wells of tritent x100 in 1 tube

all tubes placed in ice

Centrifuged 1-1½ mins Max speed

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after centrifuge

Ctl in 2 wells	50µl in each 2wells well	2wells	3 times for reliability
Trit in 2wells	2wells	2wells	
DMSO			
0.2			

- tubes were put into a box
- plates were diluted with 50µl of substrate mix agent that captures the released enzymes
- Sticker placed over plate
- incubation for 30mins
- add 50µl of ~~substrate mix again~~ stop solution to stop the reaction
- read OD value neg cont ≈ 100% safe
  pos contrl ≈ 1.9 100% toxic

BRING USB TO COLLECT DATA TOMORROW

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Day 9

- alamarBlue Assay - to see live bacteria
- crystal violet Assay - like done before
- create plates of planktonic bacteria
- LDH Assay (48 hr)
- live/dead assay

alamarBlue is light sensitive (dark plates)

AB
- aspirated biofilm plates twice w/ saline
- leave to dry for 5-10 mins
- tube in alfoil
- lights off on hood } bc. ab is light sensitive

ab: broth
1:10 dilution

10.4 : 93.6 mL
mL

200 µL ab to each well, broth column first

two tubes of ab bc. 50 mL in each tube not enough

LD staining
- buffer that fixs the biofilm, biofilm that are dead remain dead, alive remain alive
- solution out of freezer, placed in cold water
- Chamber plates same 2x aspiration 350µL saline
- 1:5
- 2mL 8mL buffer water
- glutaraldehyde solution
- 200µL in each chamber
- wait for 45mins (chamber slides)

ab
- after 1 hrs ab: read and get results on fluorescent microplate reader

alamarBlue incubating and checking w/ fluorescent reader every 1 hr because the biofilms matrix prevents the dye from entering the bacterial cells immediately. We are observing at intervals of 1 hr to for the dye reach through the matrix and into the live bacteria

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LD Staining

Chamber plates 3x wash with MilliQ water

1 dye
1 ×22.5µl each
22.5µl in 15 mL MQ water both dye together

Live/Dead Staining in dark
because dyes are sensitive

seeing live bacteria: Sytop dye

dead bacteria: PI dye

200µl of mixture in each chamber

Incubate for 15

~~Wash~~ aspirate dye

wash wy M water 1x

take chambers off the slides place cover slips over slides
add mounting ari to slides (prevent bioffilm from drying)

nail polish as a 'glue' to seal the cover plates

Slides placed under cover

LDH Assay 48hr

just like 24hr

Tonight;
- alamarBlue
- Live/D
- Phage Titre

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cell media

with penicillin

anti biotic

10% FBS - which was trypsin

trypsin was added before 10 min incubation to detach the cell the collagen coated flask

CRYSTAL V. ASSAY NOTE:

- after treatment of CV and incubation 15mins, aspirate with MilliQ water.
- dried overnight, why used? to absorb CV
- 180µl/well acetic acid
- ~~incubate~~ shake on plate shaker (2hr)
- absorbance at 595nm OD values

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1. cytotoxicity

bar graph

24hr

y - OD value

x - treatments

-ve

+ve control

48hr

Fig 5

2. planktonic

false	Cu	Cu+c. Ph	Ph
A1	70.8		(-)
A2			(-)
:			(-)
-			
-			

sensitivity

3. Biofilm

① crystal violet

bar graph

② Cryst V

OD

0.2

③ 4D staining images

AB

- measures viability and growth, metabolic rate over time

CV

- measures biomass of the bacterial biofilm

- the more stars, more inhibitory effect against what is presented by the +ve control

- smaller the T line, less of deviation from median
larger, more variation

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Live/Dead
viewing under the confocal microscope

SYTO - green