Project Report 190417, Measuring antimicrobial effect on samples from Pharma Lundensis.

Summary

Spago Nanomedical AB has analysed four samples (1, 4, 5 and 6) from PharmaLundensis by testing them for inhibitory effect on *E.coli* and yeast (*Saccharomyces cerevisiae*) by comparison of the inhibitory effect on microbial growth of samples and antibiotics standards by the plating method. It involves culturing the microorganisms, first in a liquid medium and then spreading small samples evenly on a gel medium where the microorganisms normally grow quickly and form a lawn of colonies visible to the eye. To test the effects of antibiotics small test discs of paper are placed on top of the medium and after some time the plates are inspected. If the bacterial growth has been inhibited by the antibiotics, a clear zone can be seen around the discs. Thus unknown samples can be compared for their antibiotic effect.

First, the four samples were tested untreated. Only sample 6 (Avlopp m. Antibiotika) showed an inhibitory effect on *E.coli.* Sample 1 is a blank, which, as expected, showed no inhibitory effect. Next, all samples were concentrated by a factor of 100 and compared both before and after concentration.

After concentration there was still no sign of an inhibitory effect from samples 1, 4 and 5. It is concluded that samples 1, 4 and 5 cannot contain more than 0.1 % of the inhibitory content of sample 6.

Materials and Methods

Acronyms:

AP	Ampicillin
CIP	Ciprofloxacin
IMI	Imipinem
MZ	Metronidazole
T	Tetracyclin
VA	Vancomycin
AMB	Amphotericin
OD_{600}	Optical density at 600 nm

Samples from Pharmalundensis

1. ”Prov 1. Avloppsvatten utan antibiotika men 1 % salt. Avloppsvatten”

4. ”Prov 4. Kondensat första 500 mL. Från Avlopp med Antibiotika. Första Kond. m Antib.”

5. ”Prov 5. Kondensat” mitt-fraktion efterföljande 500 mL. Från Avlopp med Antibiotika. Mitt-frak kond Med Antib.”

6. ”Prov 6. Avloppsvatten MED antibiotika enl mail 190301. 6.”B” Avlopp m. Antibiotika”

1c. Sample 1 concentrated 100-fold by evaporation.

4c. Sample 4 concentrated 100-fold by evaporation.

5c. Sample 5 concentrated 100-fold by evaporation.

6c. Sample 6 concentrated 100-fold by evaporation.

Living material-Bacteria and yeast

The *E.coli* strain was isolated locally. The yeast (*Saccharomyces cerevisiae* ATCC 9763) was obtained from Sigma Aldrich.

Other materials

Liquid medium for both organisms were from VWR (LB Miller broth for bacteria and Sabouraud for yeast). Solid medium (LB agar Miller and Sabouraud 2% dextros-agar) were also from VWR.

Discs (diameter of 6 mm) with and without antibiotics were obtained from Mast Group via Nordic Biolabs. The following discs were used; Ampicillin (AP, 10), Ciprofloxacin (CIP, 5), Imipinem (IMI, 10), Metronidazole (MZ, 2.5), Tetracyclin (T, 10), Vancomycin (VA, 5) and Amphotericin (AMB, 20). Abbreviations and their amounts in µg are given within parenthesis.

A UV-1800 (Shimadsu) spectrophotometer was used to measure the growth of the overnight cultures. A rotavapor RII from Buchi was used to concentrate samples in 250 or 500 mL flasks.

Culturing of bacteria and yeast

Both organisms were kept at RT. They were re-streaked every week in order to be ready for fast growth-start on liquid media. Liquid cultures were grown overnight at Room temperature with low speed vortexing (240 rpm) and the OD_{600} of the cultures were measured.

Plating of bacteria and yeast

Overnight cultures were measured (diluted to 0.1, 0.2 or 0.3 OD_{600}) in sterile 0.9% NaCl. Diluted culture was distributed over the plate with a metal plate spreader. Between 0.1 and 0.2 mL of the dilution was spread (see appendix for details).

Application of discs

About one hr after spreading, discs were placed on the plates using metal tweezers. Discs with antibiotics was taken directly from their package (cartridge). For samples to be investigated, 15 µL of sample was added to blanc-discs few minutes before application on plates. Duplicate plates were made for samples (1, 4, 5 and 6). Duplicate plates of controls were only made in the first two screenings. The plates were incubated at 37°C overnight or at room temperature over the weekend.

Results

Sensitivity of the organisms to antibiotics.

Inhibition zones for *E.coli* was observed for Ampicillin (AP), Ciprofloxacin (CIP), Imipinem (IMI) and Tetracyclin (T) but not for Metronidazole (MZ) and Vancomycin (VA). Inhibition zones of AMB were observed for yeast.

Inhibitory effect of samples from Pharmalundensis.

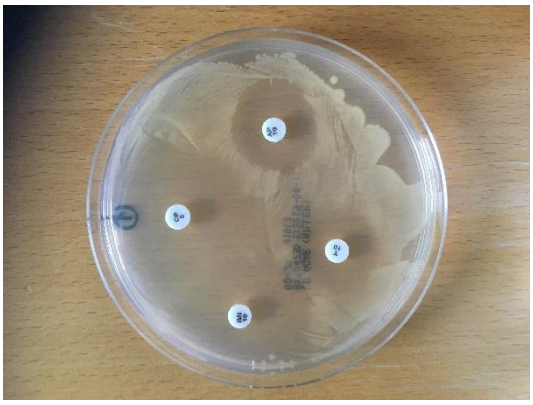
The four samples were first examined together (190315). Only sample 6 gave rise to an inhibition zone on plates with *E.coli.* The reference sample 1 was therefore concentrated 100-fold by evaporation. Next, sample 1, concentrated sample 1 and sample 6 were compared (Plating 190404). Again only sample 6 gave rise to an inhibition zone. Finally, the remaining three samples (4, 5 and 6) were concentrated and all four samples (before and after concentration) were compared for their ability to inhibit growth (plating 190412). Only sample 6, with or without concentration gave rise to inhibition zone.

The different property of sample 6 is evident. An inhibition zone is seen around it, whereas for the other samples bacteria grow tightly around the disc, even after a 100-fold concentration. Judged from un-concentrated samples, a conservative estimate would be that the three samples contain a 10-fold lower amount of inhibiting substance. Concentration of the samples (1, 4 and 5) by a factor of 100 suggests that the difference is 100-fold stronger, *i.e* there is a 1000-fold difference between sample 6 and the other three samples.

On plates with yeast, no inhibition zones from the four samples were observed.

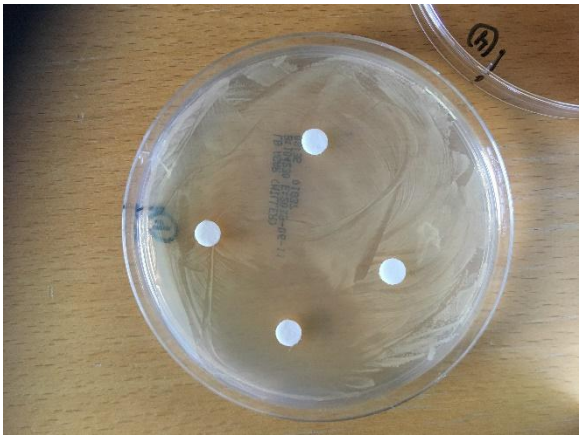
Selected figures from plating 190404 and 190412

Plate nr 7 from 190404. Samples 1, 1c and 6, clockwise. Only sample 6 gives an inhibition zone.



E.coli plate 1 (190412). From left, CIP, AP, T and IMI, clockwise. The four antibiotics give inhibition zones.

E.coli plate 3 (190412). From left, samples 1, 4, 5 and 6, clockwise. Only sample 6 gives inhibition.



E.coli plate 4 (190412). From left, samples 1c, 4c, 5c and 6c, clockwise. Only sample 6c gives inhibition.

APPENDIX

Notes: Plating of cells 190315, 190404 and 190412 (Konsultuppdrag/Pharmalundensis 2019).

Plating of cells 190315 (Friday)

Started liq. cultures from plates 190313-Wednesday. Plates had been restreaked a week before. Static cultures of LB and Sabouraud liquid medium at 32 C. Only little growth seen next morning. In the afternoon much better. Left the cultures at RT from Thursday to Friday.

OD 600 measured. Diluted 1:10. After dil. Compensation, 1.26 of *E.coli* and 3.1 of *S.c.* culture.

Dilution for plating: *E.coli* 1.3 mL up to 13 mL with sterile 0.9%. NaCl. Yeast ; 100 µL to 3 mL. Thus, OD in the dilutions approx. 0.1-0.12. 100 µL to plate in description (from Project Report 2014) but decided to instead plate 200 µL because plates were very dry and shrunken.

SAMPLES

1. Avloppsvatten utan antibiotika med 1% salt
4. Kondensat första 500 mL. Från Avlopp med Antibiotika
5.”Kondensat” från Avlopp med Antibiotika
6. Avloppsvatten MED antibiotika enl mail 190306 (6B)

Plated 200 µL. Three plates in a row. >1 hr between plating and addition of discs. 15 µL pipetted to blank discs.

***E.coli* plates:**

Plates 1 and 2: MZ (Metronidazole), Vanco (VA), Ciprofloxacin (CIP)

Plates 3 and 4. IMI (Imipenem), T, and AP.

Plates 5 and 6. No discs added. Controls of plating. (result see above)

Plates 7 and 8. MZ, VA, CIP. Difference to 1 and 2. Faster plating of cells

Plates 9 and 10. IMI, T, and AP. Faster plating compared to 3 and 4.

Plates 11, 12, 13, 14, and a little later 15, 16 and 17. Samples 1, 4, 5 and 6 clockwise. (Plate 11 failed.)

Yeast plates

1, 2, and 3: Samples 1, 4, 5 and 6 clockwise.

4 and 5. Amphotericin (AMB) twice and blank discs twice.

Monday (190318). Generally bad spreading. Lawn not complete in the periphery. Single colonies seen.

Results *E.coli*

1 and 2, 7 and 8. CIP gives inhibition. Not VA and MZ.

3 and 4. 9 and 10. All three give inhibition zones. That is IMI, T and AP.

12, 13, 14, 15, 16 and 17 (11 lost), Last in circle **sample nr 6 has inhibition zone.**

Results YEAST

Plates 1, 2 and 3. No inhibition from any of the four samples.

4 and 5. AMB gives inhibition but the zone is not wide (blank discs OK, *i.e* no inhib.)

Plating of cells 190404 (Thursday)-Results 190405

Liquid cultures

Started liq. cultures (*E.coli* and yeast (*S.c.*)) from plates 190403 (Wednesday). (In advance, shaking 24 hrs at RT found suitable, started from plates, stored at RT, re-streaked, not more than one and a half week before). Cultures of LB and Sabouraud. OD_{600} measured. Diluted 1:10. After dilution compensation, 4.9 in *E.coli* and 5.1 for *S.c.* culture. Approximated as OD 5.0 for both.

Dilution for plating for both organisms. First 1:25 dilution to 0.2 OD. 0.2 mL O.N. culture to 4.8 mL 0.9% NaCl. Further 1:2 dilution to 0.1 OD (2mL + 2mL). Both dilutions used. 100 µL of OD 0.1 and 200 µL of OD 0.2. (4-fold difference). These two plating densities were called 0.1 and 0.4. Fresh plates used in contrast till previously (190315). Bacteria and yeast plated about an hr before adding discs.

SAMPLES

1. Avloppsvatten utan antibiotika med 1% salt (also 190315)

1c. Sample 1 concentrated 100-fold by evaporation by PL

6. Avloppsvatten MED antibiotika enl mail 190301 (6B) (also 190315)

15 µL of the samples added to blank discs. This volume was quickly absorbed and after few minutes discs were placed on the plates. On each plate 1, 1c and 6 placed clockwise.

E.coli plates with plating controls, samples and antibiotics

Nr	Aim	Plated	On Discs	Result
1	Plating control-cells	0.1	None	OK, *i.e.* good spread
2	Plating control-cells	0.4	None	OK, *i.e.* good spread
3	Plating control-NaCl	0.2 mL	None	OK, *i.e.* no growth
4	Samples 1, 1c and 6	0.1	1, 1c and 6	Only inhibition zone around sample 6
5	Samples 1, 1c and 6	0.1	1, 1c and 6	As above
6	Samples 1, 1c and 6	0.4	1, 1c and 6	As above
7	Samples 1, 1c and 6	0.4	1, 1c and 6	As abover
8	Test of sensitivity	0.1	MZ, CIP and VA	Sensitivity to CIP
9	Test of sensitivity	0.1	IMI, AP and T	Sensitivity to all three
10	Test of sensitivity	0.1	MZ, CIP and VA	Sensitivity to CIP
11	Test of sensitivity	0.1	IMI, AP and T	Sensitivity to all three
12	Test of sensitivity	0.4	MZ, CIP and VA	Sensitivity to CIP
13	Test of sensitivity	0.4	IMI, AP and T	Sensitivity to all three
14	Test of sensitivity	0.4	MZ, CIP and VA	Sensitivity to CIP
15	Test of sensitivity	0.4	IMI, AP and T	Sensitivity to all three

Yeast (_Saccharomyces cerevisiae_) plates

Nr	Aim	Plated	Discs	Result
1	Plating control	0.1	None	OK, *i.e.* good spread
2	Plating control	0.4	None	OK, *i.e.* good spread
3	Samples 1, 1c and 6	0.1	S 1, 1c and 6	No inhibition zones
4	Samples 1, 1c and 6	0.1	S 1, 1c and 6	No inhibition zones
5	Samples 1, 1c and 6	0.4	S 1, 1c and 6	No inhibition zones
6	Samples 1, 1c and 6	0.4	S 1, 1c and 6	No inhibition zones

7	Test of sensitivity	0.1	AMB (and blank)	Sensitivity AMB
8	Test of sensitivity	0.4	AMB (and blank)	Sensitivity AMB

Inhibition zones (mm) on *E.coli* summary 190408

	Sample 1	**Sample 1c**	**sample 6**	CIP	AP	IMI	T
Plate							
4	**None**	**None**	**23**				
5	**None**	**None**	**22**				
6	**None**	**None**	**21**				
7	**None**	**None**	**22**				
8				34			
9					18	25	20
10				35			
11					19	30	18
12				30			
13					16	27	17
14				30			
15					16	26	19

Result: Inhibition zone only around sample 6. Different plating density makes a small difference in the expected direction. The higher plating density (4-fold) gave slightly smaller diameter (21.5 compared to 22.5 mm).

There was a clear difference with plating density for CIP and AP. With the 4-fold higher density an average diameter of 30 mm was seen from CIP. With the lower density the diameter was 34.5 mm. The corresponding figures for AP were 16 and 18.5 mm, respectively. For T and IMI, these differences were small, although in the expected direction, of 18 and 19 mm, and 26.5 and 27.5 mm, respectively,

For yeast, an inhibition zone from AMB was observed. The zone was notably small with a diameter of only 12 mm.

Plating of cells 1904012 Results 190415

Liquid cultures

Started liq. cultures from plates 190411. Cultures, O.N., in LB and Sabouraud. OD_{600} measured. Diluted 1:10. After dilution compensation, 2.4 in *E.coli* and 4.6 for *S.c.* culture. Dilution to 0.1 for *E.coli;* 125 µL O.N. culture to 2.875 mL sterile 0.9% NaCl. Dilution to 0.3 OD for yeast; 196 µL O.N. culture to 2.8 mL sterile 0.9% NaCl. Plated 150 µL of both *E.coli* and yeast.

Bacteria and yeast plated about an hr before adding discs. 15 µL of the samples added to blanc disks. Samples 1, 4, 5 and 6 placed clockwise starting from labelling mark.

E.coli plates incubated at RT to Monday 190415

Nr	Aim	Result
1	Control: CIP, AP, T and IMI	Inhibition zones for all four
2	Samples 1,4, 5 and 6	Only sample 6. >22 mm
3	Samples 1,4, 5 and 6	Only sample 6. >25 mm
4	Samples 1c, 4c, 5c and 6c	Only sample 6c. 30-35 mm
5	Samples 1c, 4c, 5c and 6c	Only sample 6c. >30 mm
6	Plating control, just 150 µL bact. dilution	OK

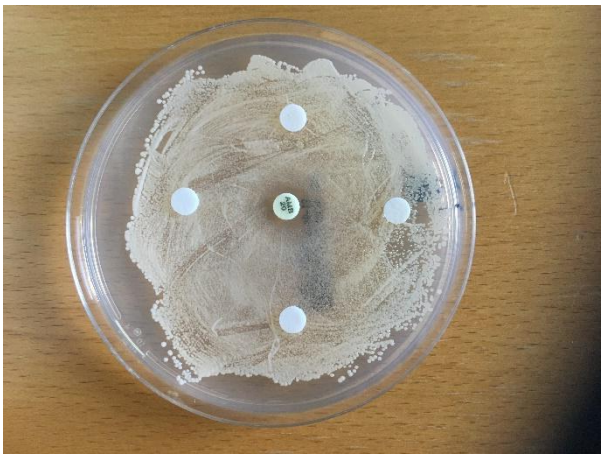
Yeast plates incubated at RT to Monday 190415

Nr	Aim	Result
AMB disc put in the middle of plates 2, 3, 4 and 5. Thus “nr 1” omitted		
2	Samples 1,4, 5 and 6	Only zone around AMB
3	Samples 1,4, 5 and 6	Only zone around AMB
4	Samples 1c, 4c, 5c and 6c	Only zone around AMB
5	Samples 1c, 4c, 5c and 6c	Only zone around AMB
6	Plating control, just 150 µL yeast dilution	OK

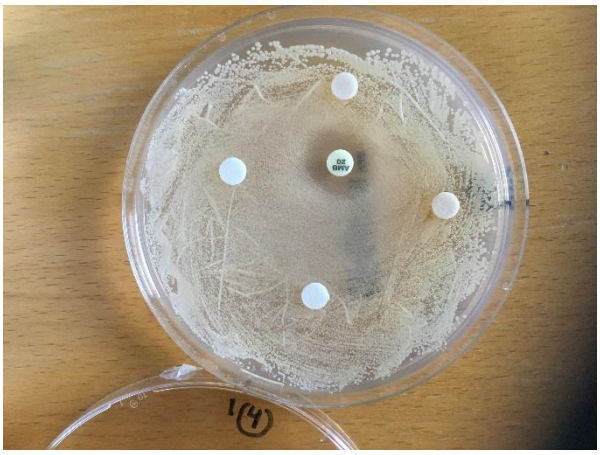
Results

Only sample 6 gave an inhibition zone on the lawn of *E.coli* before and after concentration. The concentrated sample (6c) gave a wider inhibition zone than un-concentrated sample 6.

The control plate nr 1 contains all four antibiotics. This application of four discs is too dense. CIP gives a wide zone. Inhibition zones overlap and are therefore difficult to measure.



Yeast plate 3. No inhibition from samples (not concentrated). Only AMB gives inhibition.



Yeast plate 4 with the concentrated samples. Only AMB gives inhibition