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DETERMINATION OF ANTIBIOTIC SUBSTANCES FROM ACTINOMYCETES STRAIN ST-13-2 AND ANTIBACTERIAL ACTIVITY

การบ่งชี้สารปฏิชีวนะที่ผลิตโดยเชื้อแอคติโนมัยซีตีส สายพันธุ์ ST-13-2 และฤทธิ์ในการต้านแบคทีเรีย

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ABSTRACT

The bioautographic detection of biological activity of antibiotic substances from actinomycetes strain ST-13-2 against Staphylococcus aureus *was found that they were water-soluble basic antibiotics. More than one inhibition spots were found and one of them was identical to Cloxacillin (Rf=0.26). The* in vitro *antimicrobial study of crude antibiotic solution of strain ST-13-2 was evaluated on 30 strains of each pathogenic* Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, *and* Klebsiella pneumoniae *isolated from patients. It showed a good activity against all organisms except* Ps. aeruginosa *that provided some resistibility.*

บทคัดย่อ

การศึกษาสารปฏิชีวนะของเชื้อแอคติโนมัยซีตีส สายพันธุ์ ST-13-2 โดย bioautograph ต่อเชื้อ *Staphylococcus aureus* แสดงว่าจัดอยู่ใน water-soluble basic antibiotics นอกจากนี้ สารปฏิชีวนะสามารถให้ผลการยับยั้งต่อเชื้อมากกว่า 1 จุด และพบว่ามี 1 จุดมีค่า Rf เท่ากับยาปฏิชีวนะ Cloxacillin (Rf = 0.26) ในการตรวจสอบผลของสารปฏิชีวนะของเชื้อแอคติโนมัยซีตีส สายพันธุ์ ST-13-2 ทางห้องปฏิบัติการที่มีต่อเชื้อ *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* และ *Klebsiella pneumoniae* ชนิดละ 30 ตัวอย่างแยกจากผู้ป่วยในโรงพยาบาล พบว่าให้ผลดี สามารถยับยั้งต่อเชื้อทุกตัว ยกเว้น *Pseudomonas aeruginosa* ซึ่งให้ผลเพียงบางส่วนเท่านั้น

INTRODUCTION

In the course of screening for new antibiotics from actinomycetes, the selected strain ST-13-2 was isolated from a cave soil sample collected in central region of Thailand and identified as *Streptomyces* sp. The antibiotic activity in plate assay showed superiority against most of the test organisms. Taxonomic studies indicated that strain ST-13-2 was closely related to *Streptomyces parvullus*. The liquid media with optimal pH and temperature for production by fermentation was reported. The glucose soybean medium pH 7.0 (before sterilization) at temperature 23°C was the optimum.[7]

MATERIALS AND METHODS

Determination of antibiotic substances by Thin Layer Chromatography (TLC)[3,9]

A. Preparation of antibiotics

1. Standard antibiotics[8]

The known antibiotics used as reference standards were Penicillin G, Kanamycin, Cefotaxime, Cloxacillin, Tobramycin, Bacitracin, Doxycycline, Framycetin and Erythromycin.

Concentration of each standard solution to be applied to chromatoplate was calculated from minimal inhibitory concentration (MIC) and the volume applied to each spot was about 0.02 ml.

2. Unknown antibiotics[2,7]

The *Streptomyces* strain ST-13-2 was fermented in the glucose soybean medium pH 7.0, at 23 °C which provided high yield of antibiotics in broth culture and then prepared as follows:

2.1 The whole fermentation broth (6 ml) was extracted with 3 ml of butanol, alone or in the presence of either 0.6 ml of 6N HCl or 0.5 ml of 2N NH_4OH. A 0.02 ml of each butanolic extract was applied to the chromatoplate by a micropipette.

2.2 A 10 ml of isopropanol was shaken with 6 ml of whole broth for 15 min, then centrifuged. A 0.04 ml of the supernatant was applied to the chromatoplate by a micropipette.

2.3 A 10 ml of the supernatant from the isopropanolic solution was dried *in vacuo*. The residue was triturated twice with 1 ml of ethanol. The ethanol-insoluble material was added with 2 ml of water and 2 ml of acetone. After centrifugation of the resulting suspension, a 0.02 ml of the supernatant and a 0.02 ml of the earlier ethanolic solution were applied to chromatoplates.

B. Thin Layer Chromatography[3]

1. Preparation of TLC plate

Technique	:	One way, ascending
Absorbent	:	Silica gel 60 G (7731) 30 g/60 ml of distilled water
Plate size	:	20 cm × 20 cm and 10 cm × 20 cm
Layer thickness	:	250 μm
Activation	:	Air dried for 15 min and then heating in a hot air oven at 105°C for 1 h
Distance for development	:	15 cm

2. Solvent systems for TLC[6]

The chromatoplates were developed in solvent systems as shown in Table 1.

3. Detection of compound on TLC plate[1]

3.1 Microbiological detection (bioautograph)

A Whatman no. 1 filter paper was moistened with solvent and laid on the surface of the chromatoplate and then pressed firmly with another glass plate. After 10-15 min, the paper was removed and dried then laid immediately on the inoculated *S. aureus* agar plate and left for 10-15 min. It was then removed and the agar plate was allowed to incubate for 18 h. The inhibition zones were located and their positions were marked in the corresponding places on the chromatoplate.

3.2 Chemical detection through specific colour reactions

The chromogenic reagent firstly employed was a 10% permanganate solution, followed in 10 min by the application of a 0.2% bromphenol blue solution.

Laboratory evaluation of antibiotic producing to isolated pathogenic organisms from patients[1,5]

Four groups of organisms used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* that were isolated from specimens of the patients at Siriraj Hospital and Bangkok Christian Hospital.

A. Discs and preparation of antibiotics from strain ST-13-2

1. Discs

The disc agar diffusion method was used to determine antimicrobial susceptibility test.

The standard antibiotic discs used in the test were as follows :

Amikacin	30 μg	(Biolab)	Batch.	853
Cefotaxime	30 μg	(Difco)	Lot.	732393
Ceftriazone	30 μg	(BBL)	Lot.	308565
Cloxacillin	5 μg	(BRL)	Batch.	16638
Erythromycin	15 μg	(Difco)	Lot.	724690
Gentamicin	10 μg	(BBL)	Lot.	501550
Moxalactam	30 μg	(Lilly)	Lot.	8 DG 87 A
Netilmicin	30 μg	(BBL)	Lot.	308502
Penicillin	10 Units	(Biolab)	Batch.	8462
Tobramycin	10 μg	(Lilly)	Lot.	7 RN 37 A

2. Preparation of antibiotics from strain ST-13-2

The liquid media which contained high yield of antibiotics from strain ST-13-2 was divided and treated as follows:[7]

Part 1 - The stock broth
Part 2 - Diluted the stock broth to one fold (v/v) with sterile distilled water
Part 3 - Freeze-dried (lyophilized) the stock broth and then redissolved with sterile distilled water to make 2 and 4 fold (v/v) concentration of the stock broth

B. Preparation of culture medium and plates

Melted previously prepared and steriled Muller-Hinton agar medium pH 7.2-7.4, and let cooling to 45-50°C in room temperature. Then poured into 9 cm diameter petri dishes to a depth of 4 mm (about 20 ml of medium was required in each plate). The plates were dried at 37°C for 1 h.

C. Preparation of inoculum

Thirty isolates of each group of the organisms were separately inoculated into 5 ml of soybean-casein digest broth and incubated for 6-8 h at 37°C. Each culture was standardized to match a 0.5 turbidity standard of MacFarland.

D. Inoculation of the plates

Spread 0.1 ml of each standardized inoculum over the sterile surface of the agar medium in several directions. Allowed the inoculated plates to dry at room temperature for about 5 min.

E. Assay procedure

The susceptibility discs were applied manually under aseptic precautions. Gently pressed each disc down on the surface of the inoculated agar with sterile forceps. The discs were distributed to each plate in the manner that they were sufficiently separated from one another to avoid overlaping zones of inhibition. The standard antibiotic discs tested for each organism were shown in Table 2.

Four stainless cylinder cups (0.6 cm $\times$ 1 cm) for applying the various concentration of the antibiotics from strain ST-13-2 were also placed on each disc agar plate. The cylinder cups were filled with the solution by a micropipette.

The plates were left at room temperature for 30 min, then incubated at 37°C for 16-18 h.

F. Interpretation of the test results

The diameter of each inhibition zone was measured with a sliding calipers to the nearest millimetre, reading to the point of complete inhibition as judged by the unaided eye from the underside of the plate. The zone diameter interpretative standards of NCCLS[8] (the National Committee for Clinical Laboratory Standard) was used as shown in Table 3.

RESULTS

Determination of antibiotic substances by TLC

The locations of inhibition and colours of the spots were recorded after each treatment. The Rf values were determined from the chromatoplate.

$$Rf = \frac{\text{distance of spot moving from starting point}}{\text{distance of solvent front from starting point}}$$

A. Microbiological detection

The solvent system was varied with the type of antibiotics. For separating water-soluble basic antibiotics, the solvent systems used were propanol : pyridine : acetic acid : water (15:10:3:10) and chloroform : methanol : 17% ammonia (2:1:1). Peptide antibiotics were separated by employing butanol : acetic acid : water (3:1:1) as the solvent system. Polyene antibiotics were separated by using ethanol : Conc. ammonia : water (8:1:1), and for nucleoside antibiotics the solvent was ethyl acetate : methanol (100:15). Antibiotics of macrolide group were separated by using any of three solvent systems which were ethanol : Conc. ammonia : water (8:1:1), butanol : acetic acid : water (3:1:1) and ethanol : water (4:1).

Results of bioautography were presented only from the chromatoplates that were developed in solvent system 3 (propanol : pyridine : acetic acid : water = 15:10:3:10). According to solvent systems it showed that antibiotic substances from strain ST-13-2 were water-soluble basic antibiotics. The crude fermented broth and the extraction with butanol and isopropanol provided more than one inhibition spots. The extraction with acetone and ethanol did not provide any inhibition spot. The bioautographic profile of standard antibiotics, Penicillin G and Kanamycin were also shown in Figure 1.

B. Chemical detection

The application of chromatogenic reagents to the chromatoplate yielded the colour spots shown in Figure 2. These spots did not show any related locations to the inhibition zones of antibiotic substances from strain ST-13-2, but they showed only one in the area of standard antibiotics.

According to primary identification of unknown antibiotic substances from strain ST-13-2, more standard antibiotics were applied to the chromatoplate. The results of bioautograph were shown in Figure 3. The Rf value of unknown antibiotic substances from crude fermented broth showed one inhibition spot that identical to standard antibiotic, Cloxacillin (Rf = 0.26).

Laboratory evaluation of antibiotics from strain ST-13-2

Thirty isolates of each of the four pathogenic organisms derived from the patients were used for testing of antimicrobial susceptibility with 4 dilutions of antibiotic producing strain ST-13-2 broth compared to other 4 discs of known antimicrobial agents. The results of the tests against *S. aureus*, *E. coli*, *Ps. aeruginosa* and *K. pneumoniae* were shown in Table 4, 5, 6 and 7 respectively.

The applied 4 dilutions of fermented broth from strain ST-13-2 were as follows:

U_1 = Diluted to two-fold (v/v) of the stock broth
U_2 = The stock broth
U_3 = Concentrated to two-fold (v/v) of the stock broth
U_4 = Concentrated to four-fold (v/v) of the stock broth

DISCUSSION

The antibiotic substances of strain ST-13-2 were determined by Thin Layer Chromatography. The results of bioautography using *S. aureus* showed that they were in water-soluble basic group.[2,6] The Rf values were not identical to Penicillin, Kanamycin and other standard known as antibiotics used except Cloxacillin. The Rf value of Cloxacillin was 0.26 in this study. The chemical detection test on chromatoplate provided no information about the biological activity of the components, this was only through comparison with the bioautograph.

In the laboratory evaluation of antibiotics from strain ST-13-2, the disc-susceptibility of 4 pathogenic organisms, using 30 isolates of each was summarized in Table 8 and the range of inhibition zones provided from the various dilutions of fermented broth from strain ST-13-2 was shown in Table 9.

The pathogenic bacteria used in this work were common causative agents of infectious diseases found in the hospitals. The strains of *S. aureus*, *E. coli*, *Ps. aeruginosa*, and *K. pneumoniae* obtained for this study showed percentages of sensitivity to Cefotaxime as 63.0, 40.0, 6.7 and 6.7 respectively. All isolated strains of *S. aureus* were Penicillin G resistant.

The susceptibility test of antibiotics from the fermented broth of strain ST-13-2 increased the range of inhibition zone when higher concentrated broth dilutions were employed. They were able to inhibit all isolates of *S. aureus*, *E. coli* and *K. pneumoniae* while they inhibited only some isolates of *Ps. aeruginosa*. However, the result of the experiment showed that the antibiotics could not well sterilize *Ps. aeruginosa*. So the chemotherapy of *Ps. Aeruginosa* that causes the serious infection, is still a great problem.

The rather broad spectrum of antibiotics from strain ST-13-2 is still very interesting. Even if the result of the experiment did not complete, it is also suggested that the followings should be further studied.

1. The antibiotic should be extensively tested with other kinds of microorganisms such as yeasts and molds.
2. The extraction of antibiotics should be experimented by low polarity organic solvents.
3. In case of chemical detection, using the chemical reagents besides permanganate solution sometimes will inform certain kinds of antibiotics.
4. It would be advantageous for extraction and purification to study stability of antibiotics at various pH and temperature conditions.
5. Since there are various kinds of antibiotics, it should be experimented whether the one which inhibited *Staphylococcus aureus* the same as *E. coli.*

CONCLUSION

Determination of antibiotic substances from strain ST-13-2 by bioautograph, using *S. aureus* as test organism, showed more than one inhibition spots in developing solvent system 3, propanol : pyridine : acetic acid : water (15:10:3:10). They were classified in water-soluble basic antibiotic group. The Rf value of one inhibition spot was identical to standard antibiotic, Cloxacillin (Rf = 0.26).

In clinical laboratory evaluation, the crude antibiotic solution from strain ST-13-2 was able to inhibit all isolates of *S. aureus*, *E. coli.* and *K. pneumoniae*, but some isolates of *Ps. aeruginosa.*

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REFERENCES

1. Acar, J.F. The Disc Susceptibility Test. In Lorian, V. (ed.). Antibiotics in Laboratory Medicine. The Williams & Wilkins Company, Baltimore, 1980, 24-54.
2. Aszalos, A. and Frost, D. Thin-Layer Chromatography of Antibiotic. In Hash, J.H. (ed.). Method in Enzymology. Academic Press, New York, San Francisco, London, 1975, 187-189.
3. Bobbitt, J.M. Thin-Layer Chromatography. Reinhold Publishing Co., New York, 1963, 30-69.
4. Bohni, E. Laboratory Instruction for 1. Disc Sensitivity Testing and 2. Determination of the Minimum Inhibitory Concentration (MIC). F. Haftmann La roche Co., Ltd., 1982. (Unpublished literature)
5. Finegold, S.M. and Martin, E.J. Diagnostic Microbiology. 6th ed., The C.V. Mosby Company, St. Louis, Toronto, London, 1982, 542-550.
6. Ikekawa, T., Iwami, F., Akita, E. and Umezawa, H. Application of Thin-Layer Chromatography for Separation and Identification of Antibiotics. *J. Antibiot.*, 1963, **16**, 56-57.
7. Laorpaksa, S., Yingyong, A., Thoongsuwan, S. and Pongsopida, A. Study on Antibiotic-Producing Actinomycetes from Cave Soil in Central Region of Thailand. *J. Natl. Res. Council Thailand*, 1987, **19** (1), 61-79.
8. National Committee for Clinical Laboratory Standard. Performance Standards for Antimicrobial Disc Susceptibility Test. M2-T3. *NCCLS.*, 1983, **3** (14), 491.
9. Wallhausser, K.H. Antibiotics. In Stahl, E. (ed.). Thin Layer Chromatography. 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York, 1963, 556-577.

Table 1. Solvent systems for TLC

System	Component	Ratio
1	Butanol : acetic acid : water	3:1:1
2	Chloroform : methanol : 17% ammonia	2:1:1
3	Propanol : pyridine : acetic acid : water	15:10:3:10
4	Ethanol : Conc. ammonia : water	8:1:1
5	Ethanol : water	4:1
6	Ethylacetate : methanol	100:15

Table 2. The antibiotic discs used for each test organisms

Test organism	Antibiotic disc
S. aureus	Erythromycin Penicillin Cloxacillin Cefotaxime
E. coli	Gentamicin Tobramycin Ceftriazone Cefotaxime
Ps. aeruginosa	Netilmicin Amikacin Moxalactam Cefotaxime
K. pneumoniae	Gentamicin Tobramycin Ceftriazone Cefotaxime

Table 3. Zone diameter interpretive standards

Antimicrobial agents	Disc content	Zone diameter of inhibition (mm)		
		Resistant	Intermediate	Susceptible
Amikacin	30 μg	⩽ 14	15-16	⩾ 17
Cefotaxime	30 μg	⩽ 14	15-22	⩾ 23
Ceftriazone[3]	30 μg	⩽ 12	13-15	⩾ 16
Cloxacillin	5 μg	⩽ 10	11-12	⩾ 13
Erythromycin	15 μg	⩽ 13	13-17	⩾ 18
Gentamicin	10 μg	⩽ 12	13-14	⩾ 15
Moxalactam	30 μg	⩽ 14	15-22	⩾ 23
Netilmicin	30 μg	⩽ 12	13-14	⩾ 15
Penicillin G when testing Staphylococci	10 units	⩽ 28	—	⩾ 29
Tobramycin	10 μg	⩽ 12	13-14	⩾ 15

Table 4. The results of antimicrobial susceptibility test of *S. aureus*

No.	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Erythromycin	Penicillin G	Cloxacillin	Cefotaxime
1	A	13.0	13.9	15.2	16.0	I	R	S	S
2	A	14.6	16.0	17.7	19.2	S	R	S	S
3	B	14.5	14.5	15.8	17.0	S	R	S	S
4	A	13.0	13.5	16.5	17.0	R	R	S	S
5	A	13.0	14.2	14.8	16.3	S	R	S	I
6	A	14.0	15.6	15.6	17.0	S	R	S	I
7	A	12.1	14.0	15.6	17.7	I	R	S	I
8	A	13.0	14.6	15.8	16.3	R	R	S	S
9	A	15.0	15.8	18.5	19.4	I	R	S	I
10	A	14.4	15.7	16.9	18.0	R	R	S	S
11	A	13.8	15.5	15.5	17.7	S	R	S	S
12	A	14.3	15.8	16.0	18.2	S	R	S	I
13	A	14.4	15.4	16.3	17.4	S	R	S	S
14	A	13.6	14.8	15.3	16.4	I	R	S	I
15	A	14.0	15.0	16.0	17.2	R	R	S	S
16	B	13.2	14.1	15.6	16.2	I	R	S	I
17	B	16.4	17.6	18.1	20.0	S	R	S	S
18	B	14.6	16.0	16.2	18.0	I	R	S	I
19	A	14.2	15.0	15.5	17.6	S	R	S	S
20	B	14.0	15.1	15.1	16.1	I	R	S	I
21	B	13.8	15.0	16.1	16.6	S	R	S	S
22	B	15.0	15.2	16.0	17.4	S	R	S	S
23	A	13.7	15.5	17.0	17.1	R	R	S	S
24	A	14.2	14.7	16.4	18.0	R	R	S	S
25	A	13.7	14.5	14.7	16.0	I	R	S	I
26	B	14.2	15.1	16.0	18.0	S	R	S	S
27	A	14.0	15.0	16.2	17.4	I	R	S	I
28	A	14.1	15.7	16.8	18.4	I	R	S	S
29	A	14.0	15.1	15.1	17.0	I	R	S	S
30	A	14.0	14.2	14.2	15.8	R	R	S	S
Total number of susceptible organisms						12	0	30	19
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						40.0	0.0	100.0	63.3

N.B. Hospital A = Siriraj
B = Bangkok Christian

Table 5. The results of antimicrobial susceptibility test of *E. coli*

No.	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Gentamicin	Tobramycin	Ceftriazone	Cefotaxime
1	A	13.7	15.7	17.3	18.0	S	S	S	I
2	A	16.3	17.7	18.0	20.4	S	S	S	S
3	A	15.5	16.8	18.8	21.0	R	R	S	R
4	A	16.0	17.1	17.7	19.0	S	S	S	I
5	A	13.7	15.2	17.0	19.0	S	S	S	I
6	A	16.0	17.4	18.8	21.0	S	S	S	S
7	A	11.6	12.0	13.6	14.2	R	R	S	I
8	A	14.7	16.3	17.3	18.6	S	S	S	I
9	A	12.4	13.7	15.5	16.3	I	I	I	S
10	A	13.6	16.2	17.3	18.3	S	S	S	I
11	A	15.1	15.9	17.6	19.4	S	S	S	S
12	A	14.9	16.6	18.0	20.4	S	S	S	S
13	A	16.2	17.0	17.8	19.2	S	S	S	I
14	A	15.6	17.0	17.7	20.0	S	S	S	I
15	A	16.6	18.0	19.0	21.0	S	S	S	S
16	B	14.6	16.0	18.0	20.0	S	S	S	S
17	B	14.9	16.8	18.0	20.0	S	S	S	I
18	B	16.0	17.3	18.0	18.6	S	S	S	I
19	B	15.0	16.6	18.0	18.6	S	S	S	S
20	B	16.4	17.4	19.0	21.0	S	S	S	S
21	B	15.5	16.3	17.6	19.1	S	S	S	S
22	B	15.0	17.4	18.6	20.3	S	S	S	I
23	B	15.4	16.8	16.0	18.0	S	S	S	I
24	B	15.2	16.5	17.8	20.0	S	S	S	I
25	B	15.0	16.0	17.6	18.5	S	S	S	S
26	B	16.0	16.7	17.8	20.5	S	S	S	I
27	A	16.1	17.1	18.2	20.5	R	R	S	S
28	A	13.5	14.8	15.2	18.0	S	S	S	I
29	A	14.1	15.0	16.1	18.2	R	R	S	I
30	A	15.0	16.9	17.1	19.3	R	R	S	I
Total number of susceptible organisms						24	24	29	12
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						80.0	80.0	96.7	40.0

Table 6. The results of antimicrobial susceptibility test of *Ps. aeruginosa*

No.	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Netilmicin	Amikacin	Moxalactam	Cefotaxime
1	A	0	0	0	0	R	R	R	R
2	A	0	0	0	0	R	R	R	R
3	A	0	0	0	0	R	R	I	R
4	A	0	0	0	9.6	R	R	R	R
5	A	0	0	0	8.3	S	S	R	R
6	A	0	7.9	9.0	9.2	S	S	I	I
7	A	0	0	0	0	S	I	I	I
8	A	7.8	9.0	10.1	12.3	S	S	I	I
9	A	0	0	0	0	R	R	I	R
10	A	0	0	8.5	9.4	S	S	R	R
11	A	0	0	0	0	S	S	I	R
12	A	0	0	0	0	S	R	I	I
13	A	0	7.9	9.0	10.0	R	I	I	I
14	A	0	0	0	0	R	R	I	I
15	A	0	0	0	0	S	S	R	I
16	A	0	0	0	0	S	S	I	I
17	A	0	0	9.1	11.0	S	I	I	I
18	A	0	9.3	10.8	12.0	R	R	R	R
19	A	0	0	0	0	I	R	I	I
20	A	0	0	0	0	S	S	I	I
21	A	16.8	17.4	18.8	20.0	S	S	S	S
22	B	0	0	0	0	S	S	R	I
23	B	0	0	0	0	S	S	I	I
24	B	0	9.0	10.4	11.8	S	I	I	R
25	B	22.2	22.8	23.5	26.0	S	S	S	S
26	B	9.3	9.6	11.2	13.5	S	S	I	I
27	B	0	0	0	8.9	R	R	R	R
28	B	13.8	15.6	17.2	18.9	S	S	S	I
29	B	10.3	11.4	12.8	14.9	S	S	R	R
30	B	0	0	0	0	S	I	R	R
Total number of susceptible organisms						20	15	3	2
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						66.7	50.0	10.0	6.7

Table 7. The results of antimicrobial susceptibility test of *K. pneumoniae*

No.	Hospital	Inhibition zone in mm				Susceptibility			
		U_1	U_2	U_3	U_4	Gentamicin	Tobramycin	Ceftriazone	Cefotaxime
1	B	11.8	12.9	13.7	15.0	R	R	I	R
2	B	13.8	15.6	16.3	18.0	S	S	S	I
3	B	13.4	14.7	15.8	17.1	I	R	I	R
4	B	13.2	15.0	16.6	18.0	R	R	S	I
5	B	11.3	12.6	14.8	16.7	R	I	I	R
6	B	15.9	16.2	17.7	20.3	R	R	I	R
7	B	14.6	15.7	16.7	18.1	I	R	S	I
8	B	11.6	13.2	14.0	16.3	I	R	I	I
9	B	14.9	15.5	15.8	16.9	S	I	I	R
10	B	12.8	13.6	14.1	16.2	I	I	S	I
11	B	12.6	13.7	14.9	16.5	I	I	S	I
12	B	13.9	14.0	15.4	16.8	I	S	S	I
13	B	12.3	13.5	16.0	17.2	I	I	S	I
14	B	13.8	15.3	16.7	18.8	I	S	S	I
15	A	15.0	15.5	16.0	18.5	S	I	S	I
16	A	13.8	15.3	16.1	17.4	R	R	S	I
17	A	14.9	15.1	15.5	16.9	I	S	S	I
18	A	14.9	15.9	18.2	19.3	S	S	S	I
19	A	14.0	14.7	15.6	16.2	S	S	S	I
20	A	12.0	12.8	14.5	15.2	R	I	S	I
21	A	13.7	15.1	16.2	17.7	S	S	S	I
22	A	13.2	14.3	15.5	16.7	R	R	R	I
23	A	14.0	14.7	16.0	17.8	R	R	R	I
24	A	12.1	12.8	14.5	16.9	R	R	R	R
25	A	13.2	14.7	16.2	17.3	R	I	S	R
26	A	17.3	18.1	20.7	22.1	R	R	I	I
27	A	13.4	14.4	16.7	18.9	R	R	R	R
28	A	13.3	15.5	16.0	17.8	S	I	S	I
29	A	14.2	15.0	15.9	18.0	S	S	S	S
30	A	15.6	17.1	18.0	19.5	S	S	S	S
Total number of susceptible organisms						9	9	19	2
Total number of test organisms						30	30	30	30
Percent of susceptible organisms						30.0	30.0	63.3	6.7

Table 8. The antibiotic susceptibility test of various pathogenic organisms

Antimicrobial agents	Percentage of sensitive strains			
	S. aureus	*E. coli*	*Ps. aeruginosa*	*K. pneumoniae*
Amikacin	—	—	50.0	—
Cefotaxime	63.0	40.0	6.7	6.7
Ceftriazone	—	96.7	—	63.3
Cloxacillin	100.0	—	—	—
Erythromycin	40.0	—	—	—
Gentamicin	—	80.0	—	30.0
Moxalactam	—	—	10.0	—
Netilmicin	—	—	66.7	—
Penicillin G	0	—	—	—
Tobramycin	—	80.0	—	30.0

Table 9. The range of inhibition zone of antibiotics from strain ST-13-2 against various pathogenic organisms

Dilution	Range of inhibition zone (mm) against 30 isolates of			
	S. aureus	*E. coli*	*Ps. aeruginosa*	*K. pneumoniae*
U_1	12.1-16.4	11.6-16.6	0-22.3	11.3-17.3
U_2	13.5-17.6	12.0-18.0	0-22.8	12.6-18.1
U_3	14.2-18.5	13.6-19.0	0-23.5	13.7-20.7
U_4	15.8-20.0	14.2-20.5	0-26.0	15.0-22.1

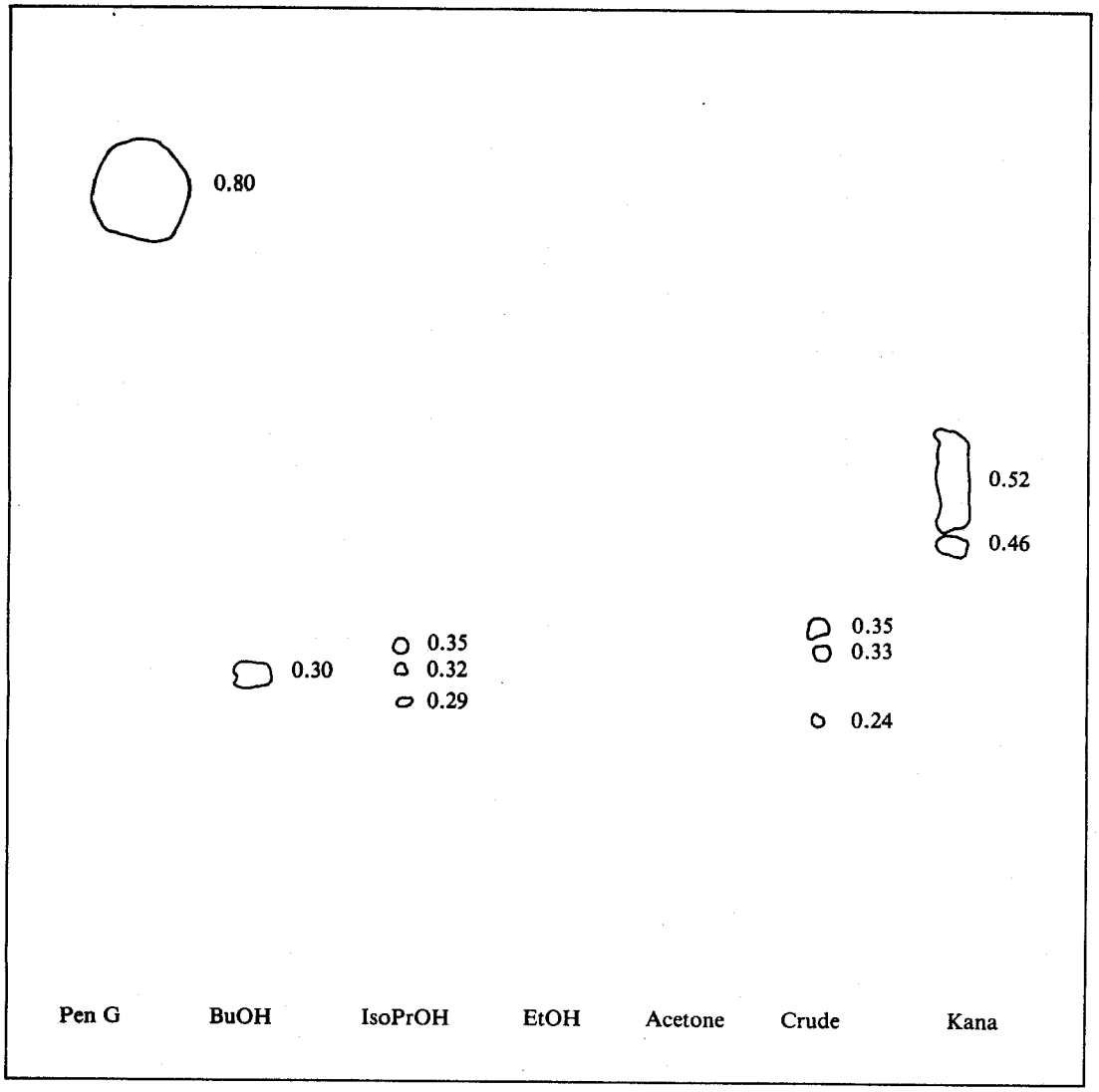

Fig. 1 Bioautographic detection of antibiotic on paper, prepared as "reprints" from Thin-Layer Chromatoplate

TLC system - Propanol : pyridine : acetic acid : water (15:10:3:10)
Organism - *S. aureus*

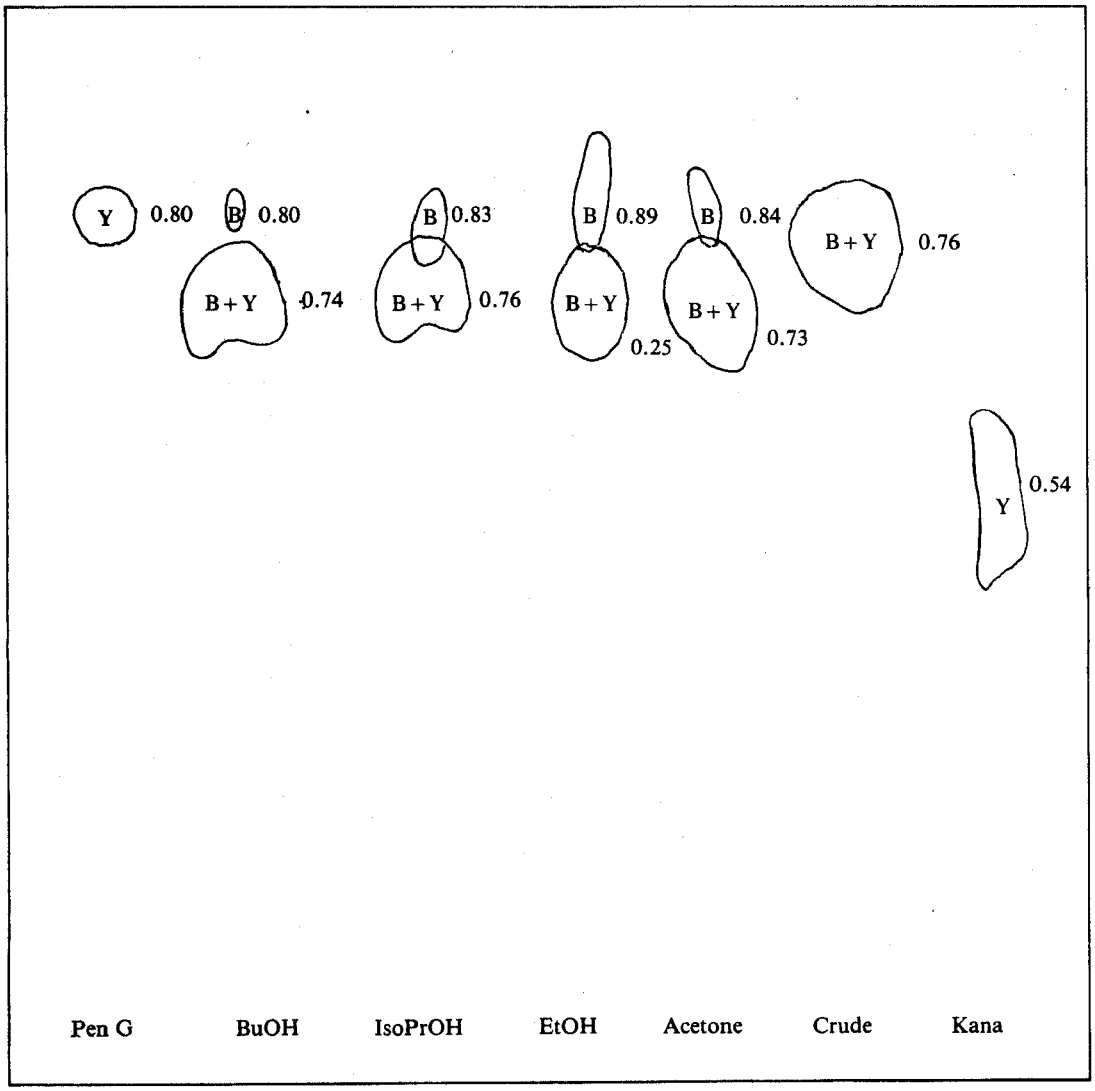

Fig. 2 Thin-Layer Chromatogram of antibiotics sprayed with 10% $KMnO_4$ solution and 0.2% bromphenol blue solution

TLC system - Propanol : pyridine : acetic acid : water (15:10:3:10)
Colour - B = Brown
- Y = Yellow

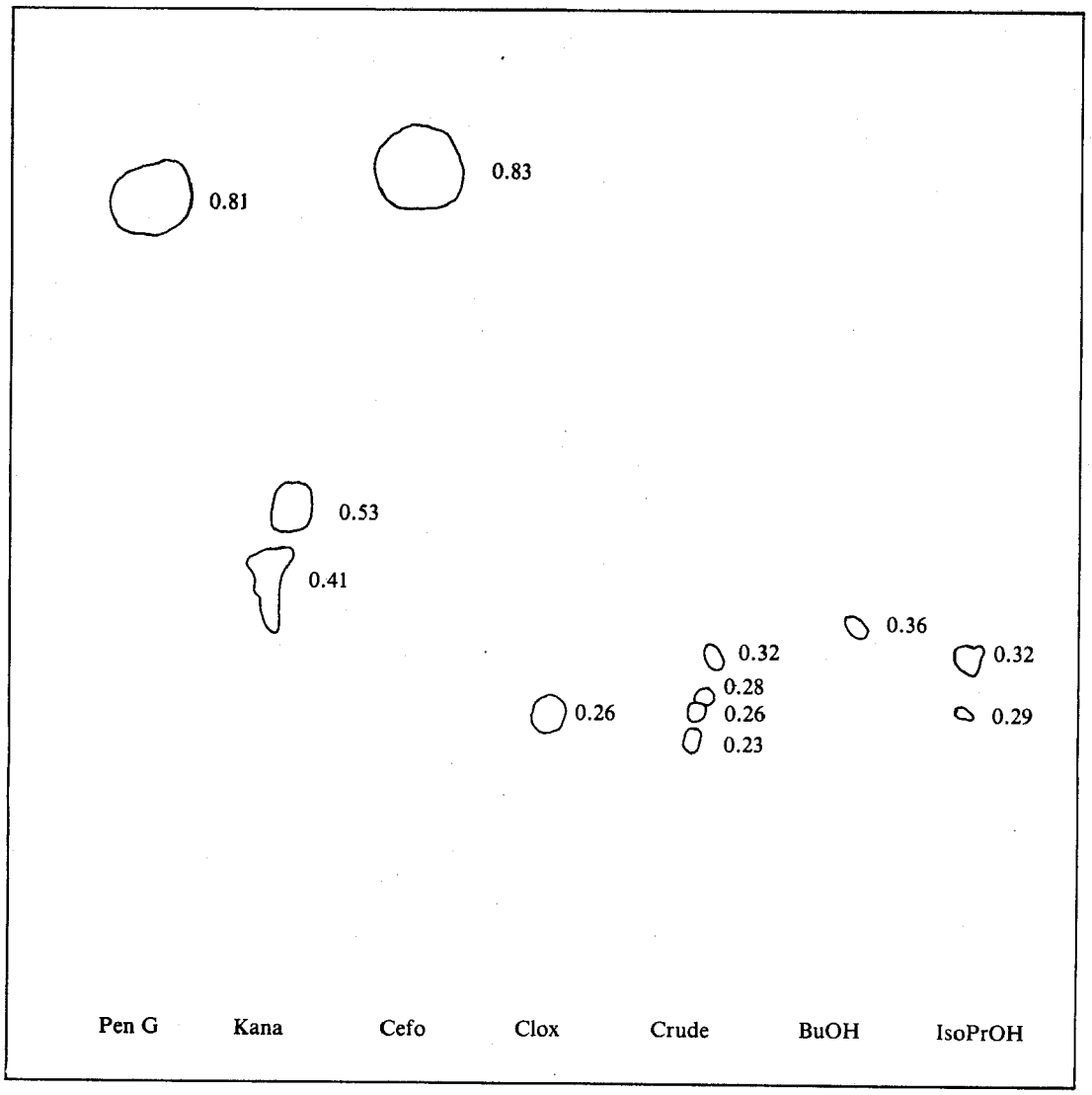

Fig. 3 Bioautographic detection for primary identification of unknown antibiotics on paper, prepared as "reprints" from Thin-Layer Chromatoplate

TLC system - Propanol : pyridine : acetic acid : water (15:10:3:10)
Organism - *S. aureus*

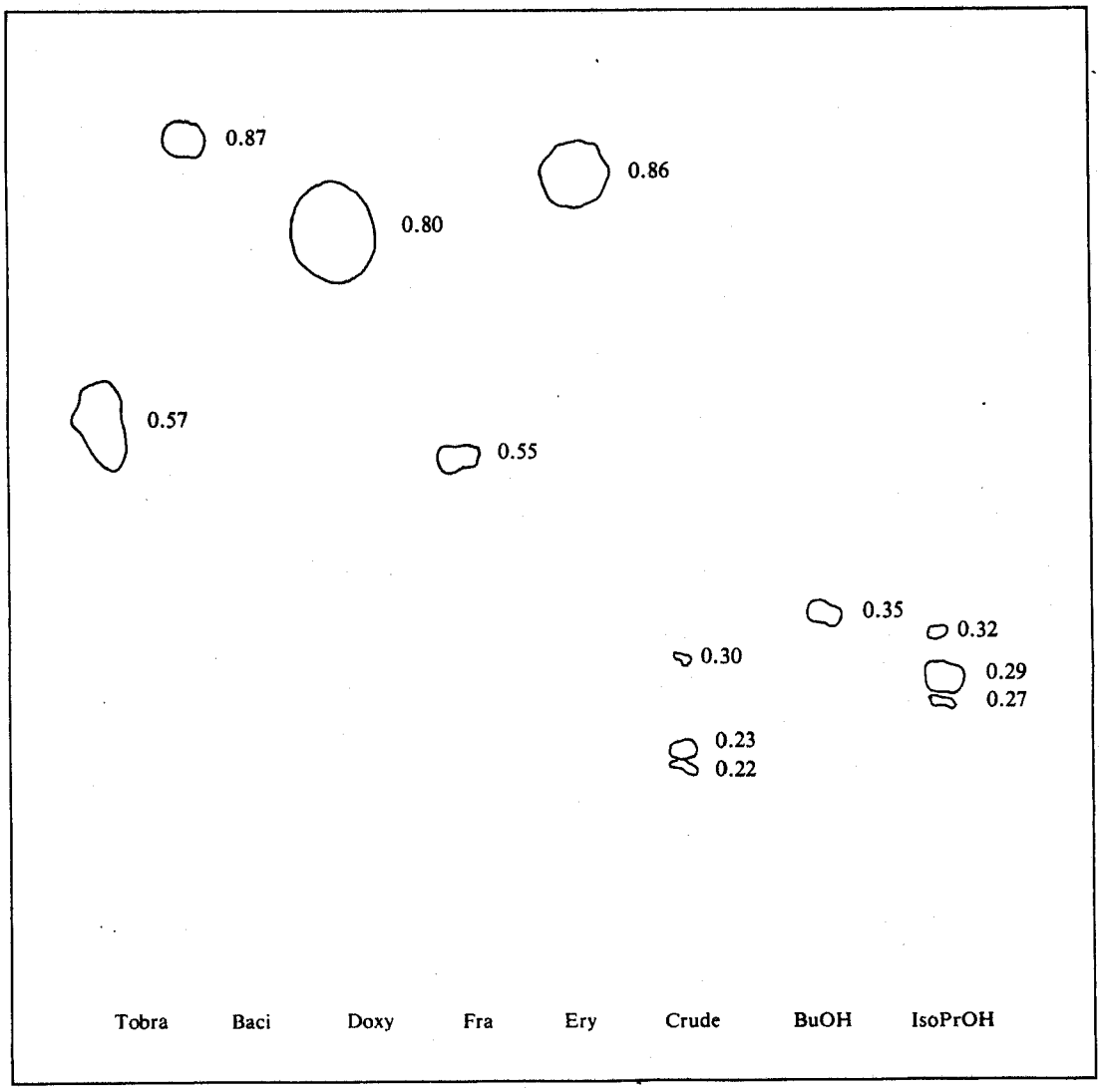

Fig. 3 (Continued)