The African Journal of MEDICAL SCIENCES

Volume 3

1972

BLACKWELL SCIENTIFIC PUBLICATIONS
Oxford London Edinburgh Melbourne

Afr. J. med. Sci. (1972) **3**, 45–51.

Antimicrobial Activities of Some *Bacillus* Species Isolated from Nigerian Soils*

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(*Revision received* 4 *January* 1971)

Summary. A simple screening technique was applied to nineteen samples of soil collected in the Ibadan area and resulted in the isolation of forty-three strains of spore-forming aerobic rods, of which twenty strains showed antimicrobial effect. Most of these possessed antifungal effect. One isolate (No. 2) strongly inhibited *Klebsiella aerogenes*, and another (No. 40) showed strong antibiotic effect on Gram-positive, Gram-negative and acid-fast bacteria, yeasts and moulds. Identification of the isolates and characterization of their antibiotic products is under investigation.

Résumé. Une methode d'essais trés simple, était applique aux dix neuf échantillons des sols recueillis de la region d'Ibadan, Nigeria. Nous avons isolé 43 types des microbes, dont 20 types montraint l'activité antimicrobienne. La plupart de ces types démonstrent aussi l'action fungicide. Un produit (No. 2) inhibité fortment *Klebsiella aerogenes*, un autre produit (No. 40) demonstre forte activité antibiotique centre les micro-organisms 'Gram-positives', 'Gram-negatives' et acid-resistantes, aussi contre les levures et les moisissures. On continue à identifier la nature chimique et les propriétés caractéristiques de ces produits antibiotiques.

INTRODUCTION

Nigerian soils have hardly been examined for antibiotic-producing bacilli; only one report has been published on a *Bacillus pumilis* strain isolated in Northern Nigeria, which produced an antibiotic named 'Pumilin', which inhibited the growth of Gram-positive bacteria (Bhate, 1955). We started a programme for screening Nigerian soils for antibiotic-producing *Bacillus* spp.

MATERIALS AND METHODS

Cultures and media

Twenty-one test organisms were used in this study. These represented four Gram-positive, eight Gram-negative and one acid-fast bacteria as well as two yeasts and six filamentous

* This paper was presented at the International Pharmaceutical Conference, London, September 1969.

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fungi. Of these, six were standard NCTC cultures (*Bacillus subtilis* 8236, *Staphylococcus aureus* 6571, *Serratia marcescens* 1377, *Klebsiella aerogenes* 5055, *Pseudomonas aeruginosa* 6750 and *Mycobacterium phlei* 8151) while the remainder were our Department's class cultures (*Sarcina lutea*, *Streptococcus faecalis*, *Proteus vulgaris*, *Shigella sonnei*, *S. schmitzii*, *Escherichia coli*, *Salmonella* sp., *Saccharomyces cerevisiae*, *Candida albicans*, *Cladosporium resinae*, *Chaetomium globosum*, *Aspergillus niger*, *A. flavus*, *Penicillium* sp. and *Mucor* sp.).

Bacteria were maintained on nutrient agar slants supplemented by 1% yeast extract and subcultured biweekly; while fungi were maintained on Sabouraud's dextrose agar and subcultured monthly. All the media used were the products of Oxoid Ltd (England).

Soil samples

Nineteen soil samples were collected in sterile vials from various soils in the Ibadan area, avoiding places excessively contaminated by humans. The samples were examined promptly but no special precautions were taken for their storage.

Isolation of the bacilli

The soil sample was mixed well, a small quantity was suspended in 10 ml of sterile distilled water and the suspension was immersed in a water bath and maintained at 70°C for 10 min to kill most of the vegetative forms and leave only sporulating micro-organisms. A large loopfull of the treated suspension was streak-plated on nutrient agar containing 1% yeast extract (pH 7). The plates were incubated at 27°C for 48 hr and the bacterial colonies were examined microscopically. Spore-forming rods were isolated and further purified by streak plating. Rough decisions were made regarding which of the colonies represented 'different' bacilli and such isolates were tested for antimicrobial activities. The isolates were maintained on slants of nutrient agar containing 1% yeast extract, and subcultured every 2 weeks. Since the antimicrobial activity of a microbial isolate may change unless it is maintained under special conditions, the isolates were tested as promptly as possible.

Testing the isolates

The cross-streak technique was followed. Standard 10-cm Petri dishes were each prepared with 15 ml of nutrient agar containing 1% yeast extract, pH 7 (for testing against bacteria) or nutrient agar containing 1% each of yeast extract and dextrose, pH 6 (for testing against fungi). The plates were pre-dried by incubation at 37°C for 6–12 hr, to avoid spreading growth by some bacilli. A 5–6 cm straight streak of the isolate to be tested was made at one side of the plate and the plates were then incubated at 27°C for 48 hr. The test organisms were then applied as 5–6 cm cross-streaks made from 24-hr-old broth suspensions (in case of bacteria and yeasts) or from a dilute suspension consisting mainly of spores (in case of moulds). The plates were re-incubated at 32°C (for bacteria) or at 25°C (for fungi), and the growth of the test organisms was observed after incubation for 24 and 48 hr (in case of bacteria) or 2 and 4 days (in case of fungi). The amount of inhibition of growth, if any, was measured as the approximate percentage of the total length of the test streak in which growth did not appear. An alternative method was sometimes followed in testing for antifungal activities. In this case, the isolate to be tested was applied as a 2-cm cross in the centre of the plate, and the test moulds were applied as half-circles placed about 4 cm away from the centre of the plate. Antifungal activities were then evident by the failure of the mould growth to advance towards the bacillus growth.

Confirmation of antibacterial activities by agar diffusion

Three streaks of each active bacillus, placed 3 cm apart, were made on plates of nutrient agar containing 1% yeast extract, pH 7. The plates were incubated at 27°C for 48 hr and then holes 8 mm in diameter were made with a sterile cork-borer in the growth-free part of the agar, adjacent to the bacterial growth. It was assumed that the plugs of agar resulting from boring contained the antimicrobial substances. To test for these, 10-cm plates were prepared each with 10 ml of nutrient agar that was allowed to harden as a base layer. This was followed with 4 ml of nutrient agar containing a small inoculum of the test bacteria as a seed layer. Four of the antibiotic-containing plugs from different isolates, were placed on each plate, and the plates were refrigerated for 4 hr to allow diffusion of the antibiotic into the agar. The plates were then incubated for 16–18 hr at the optimum growth temperature for the test bacteria, and the diameter of the zone of inhibition around each plug were measured. Antifungal activities were not confirmed by this procedure.

Identification of active isolates

The morphological, staining, cultural and a few of the biochemical characteristics of antibiotic-producing isolates were determined according to the methods outlined in Bergey's Manual. With a few of the isolates, full identification was carried out.

TABLE 1. Screening of soil samples for antibiotic-producing *Bacillus* species

Soil sample	*Bacillus* species isolated	Active isolates
I	1, 2	1, 2
II	3, 4	—
III	5, 6	5, 6
IV	7, 8, 9	7
V	10, 11	10, 11
VI	12, 13, 14	12, 13, 14
VII	15, 16	15, 16
VIII	17, 18, 19	—
IX	20	20
X	21, 22, 23, 24	21, 23
XI	25	25
XII	26, 27, 28	27
XIII	29, 30, 31	—
XIV	32	—
XV	33, 34, 35	—
XVI	36	—
XVII	37, 38, 39	39
XVIII	40, 41	40, 41
XIX	42, 43	—

RESULTS AND DISCUSSION

Forty-three aerobic, spore-forming, rod-shaped bacterial isolates were obtained. Of these, twenty showed antimicrobial effect (Table 1). The results of the tests for antimicrobial

Isolate number	Incubation (hr)	Percentage of inhibition* of:													Day of incubation	Percentage of inhibition* of:							
		B. subtilis	*S. aureus*	*S. lutea*	*S. faecalis*	*M. phlei.*	*S. marcescens*	*K. aerogenes*	*P. vulgaris*	*Salmonella* sp.	*Sh. schmitzii*	*Sh. sonnei*	*E. coli*	*Ps. aeruginosa*		*S. cerevisiae*	*C. albicans*	*Mucor* sp.	*Cl. resinae*	*Ch. globosum*	*Penicillium* sp.	*Asp. niger*	*Asp. flavus*
1	24	—	40	5	—	5	—	—	—	—	—	—	—	—	2	5	—	n.t.	—	—	n.t.	—	—
	48	—	40	—	—	—	—	—	—	—	—	—	—	—	4	5	—	n.t.	—	—	n.t.	—	—
2	24	5	10	20	5	30	—	60	—	—	15	—	—	—	2	n.t.	n.t.	n.t.	—	n.t.	n.t.	100	n.t.
	48	5	10	20	5	30	—	60	—	—	—	—	—	—	4	n.t.	n.t.	n.t.	—	n.t.	n.t.	100	n.t.
5	24	5	100	100	30	40	5	60	—	—	5	—	—	30	2	25	80	n.t.	—	20	n.t.	50	—
	48	5	100	100	15	40	5	20	—	—	—	—	—	15	4	10	40	n.t.	—	—	n.t.	30	—
6	24	—	—	60	—	—	5	—	—	—	—	—	5	—	2	n.t.	n.t.	n.t.	—	n.t.	n.t.	100	n.t.
	48	—	—	30	—	—	—	—	—	—	—	—	—	—	4	n.t.	n.t.	n.t.	—	n.t.	n.t.	100	n.t.
7	24	—	—	—	n.t.	—	—	—	—	—	—	—	—	—	2	n.t.	10	n.t.	—	—	n.t.	100	—
	48	—	—	—	n.t.	—	—	—	—	—	—	—	—	—	4	n.t.	10	n.t.	—	—	n.t.	100	—
10	24	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	2	n.t.	100	n.t.	—	50	n.t.	30	—
	48	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	4	n.t.	70	n.t.	—	20	n.t.	20	—
11	24	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	2	n.t.	100	n.t.	—	—	n.t.	25	—
	48	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	4	n.t.	70	n.t.	—	—	n.t.	10	—
12	24	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	2	n.t.	60	n.t.	—	100	n.t.	70	—
	48	n.t.	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	4	n.t.	20	n.t.	—	30	n.t.	40	—
13	24	10	20	100	5	25	5	—	—	—	—	—	60	5	2	15	50	n.t.	—	—	n.t.	70	—
	48	10	10	100	5	25	—	—	—	—	—	—	60	5	4	15	50	n.t.	—	—	n.t.	20	—
14	24	—	5	5	—	60	—	—	—	—	—	—	—	—	2	n.t.	20	10	—	—	n.t.	20	—
	48	—	5	5	—	20	—	—	—	—	—	—	—	—	4	n.t.	10	10	—	—	n.t.	10	—
15	24	60	5	60	—	40	—	—	—	—	40	n.t.	—	—	2	n.t.	—	n.t.	—	—	n.t.	—	—
	48	10	5	10	—	10	—	—	—	—	5	n.t.	—	—	4	n.t.	—	n.t.	—	—	n.t.	—	—
16	24	15	40	80	n.t.	50	n.t.	—	n.t.	—	—	—	—	—	2	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.
	48	10	40	40	n.t.	50	n.t.	—	n.t.	—	—	—	—	—	4	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.
20	24	—	—	5	n.t.	5	n.t.	—	n.t.	—	—	n.t.	—	—	2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	48	—	—	—	n.t.	—	n.t.	—	n.t.	—	—	n.t.	—	—	4	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
21	24	5	—	100	n.t.	100	n.t.	—	n.t.	—	—	n.t.	—	—	2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	48	5	—	100	n.t.	100	n.t.	—	n.t.	—	—	n.t.	—	—	4	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
23	24	60	—	—	n.t.	100	n.t.	—	n.t.	—	—	n.t.	—	—	2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	48	60	—	—	n.t.	40	n.t.	—	n.t.	—	—	n.t.	—	—	4	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
25	24	—	—	80	n.t.	80	n t	—	n t	—	—	n t	—	—	2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	48	—	—	80	n.t.	50	n.t.	—	n.t.	—	—	n.t.	—	—	4	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
27	24	100	70	100	n.t.	100	n.t.	—	n.t.	—	—	n.t.	—	—	2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	48	100	70	100	n.t.	100	n.t.	—	n.t.	—	—	n.t.	—	—	4	n t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
39	24	—	25	100	n.t.	60	n.t.	—	n.t.	—	—	—	—	—	2	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.
	48	—	20	70	n.t.	40	n.t.	—	n.t.	—	—	—	—	—	4	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.
40	24	20	80	100	n.t.	80	20	10	—	10	100	—	20	10	2	20	100	n.t.	20	n.t.	10	25	20
	48	10	50	70	n.t.	60	20	10	—	10	80	—	20	10	4	20	100	n.t.	10	n.t.	10	25	10
41	*24*	*10*	*80*	*100*	n.t.	50	—	—	—	—	20	—	—	20	2	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.
	48	*10*	*70*	*70*	n.t.	10	—	—	—	—	20	—	—	10	4	n.t.	100	n.t.	n.t.	n.t.	n.t.	100	n.t.

—, No inhibition; n.t., not tested.
* Percentage of the distance available for growth in which growth did not develop.

TABLE 3. Confirmed antibacterial spectra of isolates by agar diffusion

Isolate number	Zone of inhibition* (mm) on:								
	B. subtilis	*S. aureus*	*S. lutea*	*M. phlei*	*K. aerogenes*	*Salmonella* sp.	*Sh. schmitzii*	*E. coli*	*Ps. aeruginosa*
1	n.t.	23	0	0	n.t.	n.t.	n.t.	n.t.	n.t.
2	0	10	12	15	30	n.t.	10	n.t.	n.t.
5	0	43	46	22	12	n.t.	n.t.	n.t.	10
6	n.t.	n.t.	10	n.t.	n.t.	n.t.	n.t.	0	n.t.
13	9	15	39	19	n.t.	n.t.	n.t.	23	0
14	n.t.	0	0	22	n.t.	n.t.	n.t.	n.t.	n.t.
15	15	10	18	14	n.t.	n.t.	11	n.t.	n.t.
16	10	20	27	17	n.t.	n.t.	n.t.	n.t.	n.t.
20	n.t.	n.t.	0	0	n.t.	n.t.	n.t.	n.t.	n.t.
21	0	n.t.	23	29	n.t.	n.t.	n.t.	n.t.	n.t.
23	20	n.t.	n.t.	29	n.t.	n.t.	n.t.	n.t.	n.t.
25	n.t.	n.t.	29	22	n.t.	n.t.	n.t.	n.t.	n.t.
27	37	31	38	31	n.t.	n.t.	n.t.	n.t.	n.t.
39	n.t.	15	29	25	n.t.	n.t.	n.t.	n.t.	n.t.
40	12	42	42	32	12	13	27	14	12
41	9	31	35	21	n.t.	n.t.	13	n.t.	9

* The agar plug measured 8 mm in diameter; n.t., not tested.

TABLE 4. Summary of the antimicrobial spectra of the isolates

Activity against	Number of isolates
Gram-positive	1
Gram-positive and acid-fast	5*
Fungi	4
Acid-fast and fungi	1
Gram-positive, acid-fast and fungi	3
Gram-positive, Gram-negative, acid-fast	1
Gram-positive, Gram-negative, acid-fast and fungi	5

* These isolates were not tested for antifungal activity.

effect of active isolates on various bacteria and fungi are presented in Table 2; while those of the agar-diffusion confirmatory tests are shown in Table 3. Although no attempt was made to test all the colonies of spore-forming rods obtained, the results show a rather high incidence of antagonistic bacilli. The majority of the active isolates show a broad spectrum of activity (Table 4). It was interesting to note that thirteen of the fifteen isolates tested for antifungal activity were found to be active, especially against *Aspergillas niger* and *Candida albicans;* and that *Mycobacterium phlei* showed sensitivity to the isolates that is almost identical with Gram-positive bacteria (Table 5). On the other hand, Gram-negative bacteria were generally resistant; *Proteus vulgaris* and *Shigella sonnei* being resistant to all the isolates on which they were tested.

TABLE 5. The incidence of activity against various groups of micro-organisms*

Microbial groups	Number of antagonistic isolates
Gram-positive bacteria	15
M. phlei	14
Gram-negative bacteria	6
Fungi	13

* Five isolates were not tested for antifungal activity.

Gauze (1961) states that on the basis of studies on Soviet and Brazilian soils it could be concluded that the geographic location and altitude has a dramatic qualitative and quantitative influence on the incidence of antagonists among soil micro-organisms. For example he reported that about 50% of *Bacillus* spp. isolated from soils in central Asia proved antagonistic while the corresponding value for the soils around Moscow was only about 10% (Gauze, 1961). The high incidence of antagonists and the predominance of broad spectra amongst our isolates seems to be in line with these findings.

In general the antimicrobial spectra obtained here are not unique: similar spectra have been observed before (Korzybski, Kowszyk-Gindifer & Kurylowicz, 1967). Five of our isolates inhibited Gram-positive, Gram-negative and acid-fast bacteria and the fungi. The most interesting of these was No. 40 which strongly inhibited *Staphylococcus aureus*, *Sarcina lutea*, *Mycobacterium phlei*, *Shigella schmitzii*, and *Candida albicans* and showed some inhibition of all other test micro-organisms except *Proteus vulgaris* and *Shigella sonnei*. This isolate is tentatively identified as *Bacillus subtilis*. Isolate No. 41 showed a somewhat similar spectrum and was obtained from the same soil sample. On the other hand, at least two antibiotics have been previously reported to inhibit the same groups of micro-organisms one of them being produced by a strain of *Bacillus subtilis*. (Carvagal, 1953). The high incidence of antifungal activity among our isolates does not fit previous findings; only thirteen bacillus-produced antibiotics reported earlier possess antifungal activity (Korzybski *et al.*, 1967).

Isolate No. 2 is also interesting because it exerts its maximum effect on *Klebsiella aerogenes*, an organism that is generally resistant. Further investigation of these two isolates (No. 40 and 2) could be useful.

ACKNOWLEDGMENT

The authors wish to thank Mr S. F. Lawal, of the Department of Pharmacy for invaluable technical assistance.

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