AN ANTIBIOTIC FROM *PFEIFFERELLA WHITMORI**

By J. E. JAMESON, M.R.C.S.

From the Central Public Health Laboratory, Colindale

(With Plate 2)

In the course of routine work an attempt was made to prepare 'H' and 'O' agglutinating sera from a strain of *Pfeifferella whitmori* received from Guy's Hospital.

Considerable difficulty was met with in preparing a satisfactory flagellar suspension. Though most broth cultures of the organism contained a proportion of very active members, there always remained a majority of sluggish organisms. Repeated subculture in fluid media, cultivation at temperatures below 37° C., and passage through Craigie (1931) tubes did not appreciably improve this state of affairs, and it was therefore decided to cultivate the organism on the surface of 0·5 % nutrient agar plates.

One plate of 0·5 % agar used in the attempt to obtain full motility became accidentally contaminated with a motile aerobic spore-bearing organism, which swarmed on the plate except over a circular zone round the inoculum of *Pf. whitmori*. It was then shown that growth of the contaminating organism was inhibited *in vitro* by a diffusible factor which was present in culture filtrates of *Pf. whitmori*. This factor is referred to subsequently in this paper as 'whitmorin' and broth culture filtrates of *Pf. whitmori* as 'whitmorin broths'.

THE RANGE OF INHIBITORY ACTIVITY OF WHITMORIN

Whitmorin was found to be inhibitory *in vitro* to a range of organisms very similar to that acted on by penicillin: a list is given below of organisms which were found sensitive to whitmorin, the numbers of strains tested being shown within brackets:

Corynebacterium diphtheriae (over 50)
C. hofmanni (1)
Neisseria meningitidis (1)
N. gonorrhoeae (1)
N. pharyngis (1)
Micrococcus tetragenus (1)
Haemophilus influenzae (2)
Staphylococcus pyogenes (4)
Streptococcus pyogenes (8)
Pneumococcus (3)
Mycobacterium tuberculosis, strain H 37 RV (1)
Bacillus subtilis group (1)

Organisms insensitive to similar concentrations of whitmorin included *Salmonella*, *Shigella*, *Escherichia*, *Proteus*, *Pseudomonas* and *Streptococcus* Group D, though some strains of the last organism and of *Bact. coli* of faecal origin were found sensitive to higher concentrations.

Pl. 2, fig. 1 is a photograph of a lysed-blood serum-agar plate streaked with eight organisms. Ditches had been cut in opposite segments of the plate and filled with penicillin agar (4 units/ml.) and whitmorin broth agar (1/1) respectively: broth cultures of the organisms had been streaked between the ditches, and the plate incubated overnight at 37° C. The similarity in the ranges of bacterio-static activity of whitmorin and penicillin is illustrated.

The photograph in Pl. 2, fig. 2 is of a pour plate of *C. diphtheriae mitis* in serum agar, and shows inhibition of growth round Heatley cylinders filled with whitmorin broth. Potassium tellurite had in this particular case been incorporated in the medium to accentuate the visible demarcation of the zones of inhibition.

BACTERICIDAL ACTION OF WHITMORIN

The lethal action of whitmorin was first observed on *C. diphtheriae* when it was found that agar blocks cut from the centres of zones of growth inhibition (as shown in Fig. 2) were sterile, precautions having been taken to eliminate by dilution the effect of bacteriostatic concentrations of whitmorin which might be carried over with the agar blocks. The bactericidal action of whitmorin broth on *C. diphtheriae* was confirmed and more fully investigated by the following experiment:

One in ten and one in a thousand dilutions were made in nutrient broth at 37° C. of an overnight broth culture of *C. diphtheriae mitis*. A viable count

* Antibiotic properties of broth culture filtrates of *Pfeifferella whitmori* which are here recorded were demonstrated to the meeting of the Pathological Section of the Royal Society of Medicine held on 22 June 1948, at the Central Public Health Laboratory, Colindale Avenue, London, N.W. 9.

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was put up by the surface method of Miles & Misra (1938) on the 1/1000 dilution, and to each culture dilution was added a $\frac{1}{3}$ volume of whitmorin broth warmed to 37° C. The two diluted cultures were immediately replaced in the incubator, and further viable counts were made on them at hourly intervals for 4 hr. The initial counts were arrived at by simple calculation from the count made before the addition of whitmorin broth.

The counts are shown in Table 1, and are expressed in viable organisms per millilitre.

Table 1. *Bactericidal action of whitmorin on* Corynebacterium diphtheriae

(Hourly viable counts)

Time (hr.)	0	1	2	3	4
Series 1/10	470,000	440,000	263,000	200,000	156,000
Series 1/1000	4700	2800	1400	1300	1200

After 4 hr., two-thirds of the initial count of just under 500,000 organisms had been killed, while when the initial count was a little less than 5000, only three-quarters of the organisms were killed in the same time. Here again a close analogy with the action of penicillin can be drawn, in that the rate of killing was but little altered by a hundred-fold variation in the antibiotic/organism concentration ratio.

LABORATORY PREPARATION OF WHITMORIN

Once whitmorin broth was shown to have antibiotic properties, means were sought of obtaining preparations of sufficient potency for experimental purposes. A few simple tests were carried out, from which the following conclusions were drawn.

(*a*) *Optimal period of incubation*

In nutrient broth at 37° C. a maximal titre of whitmorin was reached by about the sixth day after inoculation with Guy's strain *Pf. whitmori*, after which the titre of whitmorin remained stationary or declined slowly. Surface growth on nutrient agar at 37° C. resulted in a more potent and more rapid elaboration of whitmorin which diffused into the agar, and could be extracted with distilled water, or by alternate freezing and thawing of the agar by the method recommended by Maxted (1948) in the preparation of his lytic enzyme from *Streptomyces albus*.

(*b*) *Choice of culture medium*

The only media tested were infusion agar, Todd Hewitt broth, Hedley Wright broth, Hartley broth, peptone water and Dubos's (1947) liquid medium for *M. tuberculosis*. Ordinary peptone water gave as high a yield as the various broths, between which there was little difference. The yield in Dubos's medium however was extremely poor, though growth of *Pf. whitmori* was profuse in this medium.

(*c*) *Bacterial variation in antibiotic productivity*

Six strains of *Pf. whitmori*, kindly supplied by the Curator of the N.C.T.C., were investigated for whitmorin production. Of these, two strains, 4845 and 6700, gave considerably higher yields than the Guy's Hospital strain, two gave yields similar to the Guy's strain, and two gave lower yields: one of the last two, a very old laboratory strain, gave no yield. Six colonies of *Pf. whitmori* N.C.T.C. 4845 picked from a blood agar plate and grown at 37° C. for 7 days in test-tubes of nutrient broth at equal depths gave a fivefold variation in titre between maximum and minimum yields. The relative yields from the same six colonies on two further subcultures, each for 1 week, remained substantially unaltered, though minor variations had taken place in the respective yields of the subcultures.

(*d*) *Sterilization of whitmorin preparations*

It was found that whitmorin broth retained its antibiotic potency well if heated to 100° C. for a few minutes, but not when boiled for a period of 30 min.; its bacteriostatic property was completely destroyed by autoclaving.

Whitmorin broth was rendered safe for use, after clarification by centrifugation or by Seitz filtration, by heating for 10 min. at 75° C. Preparations so treated maintained their potency for several months in the refrigerator, but not at 37° C. A sample of whitmorin broth kept at 37° C. for 72 hr. lost 55 % of its potency. All preparations of whitmorin were tested for sterility before use.

(*e*) *Methods of concentration and purification*

Concentration and purification of whitmorin was attempted only on a very small scale. It was found that whitmorin could be adsorbed on charcoal from broth culture filtrates and that it could with difficulty be eluted both by acetone and by methanol. It could be extracted from broth by butyl alcohol, though this solvent had not a favourable partition coefficient when used with slightly alkaline broth. Whitmorin is sparingly soluble in chloroform, insoluble in ether, benzene, nitrobenzene and xylene.

INHIBITORY ACTION OF WHITMORIN ON GROWTH OF *MYCOBACTERIUM TUBERCULOSIS*

Seitz-filtered whitmorin broth was diluted serially with Dubos's liquid medium for *M. tuberculosis*, doubling dilutions being put up from 1/5. A normal control, and controls containing equivalent dilutions of ordinary broth, were also prepared. Dilutions and controls were set up in 2 c.c. lots in small screw-cap bottles and to each bottle was added 0·02 c.c. of a diffuse 10-day culture in Dubos's medium of *M. tuberculosis* H 37 RV. After 6 days' incubation at 37° C. visible growth had taken place in all controls and in whitmorin dilutions above 1/40, but not in the 1/40 and lower dilutions: after a further period of 4 days the turbidity of the controls had increased, and only the 1/5 dilution of whitmorin remained completely clear: partial inhibition of growth was still apparent in the 1/10 and 1/20 dilutions.

THERAPEUTIC POTENTIALITY OF WHITMORIN

In a preliminary experiment, three consecutive daily intraperitoneal injections of 1 c.c. of whitmorin broth were found to produce no apparent ill effects in four mice. The same batch of whitmorin broth, which was a 6-day broth culture of *Pf. whitmori* N.C.T.C. 4845, seitz filtered and heated to 75° C., was used in a small-scale therapeutic trial in experimental guinea-pig diphtheria.

Sixteen 3-month-old guinea-pigs weighing 160–200 g. each were divided at random into four groups each of four animals. Each group comprised two 'treated' and two control pigs. The test organism was a virulent strain of *C. diphtheriae mitis*, cultured overnight in nutrient broth at 37° C., centrifuged and resuspended in broth. A viable count was carried out immediately before inoculation. Throughout the experiment control groups received identical treatment with 'treated' groups, the only difference in treatment being the injection of sterile nutrient broth whenever whitmorin broth was given to the test groups.

The four groups received the following doses of organisms, and treatment:

Group 1. Intraperitoneal inoculation of $1\frac{1}{4}$ million *C. diphtheriae mitis* in 0·5 c.c. of broth, followed 30 min. later by 5 c.c. of whitmorin broth intraperitoneally, and 24 hr. later by a further 5 c.c. of whitmorin broth intraperitoneally.

Group 2. Inoculum as in Group I, but given subcutaneously: treatment as in Group 1.

Group 3. Intraperitoneal inoculation of $6\frac{3}{4}$ million *C. diphtheriae mitis* in 1·0 c.c. of broth, followed 30 min. later by a single injection of 10 c.c. whitmorin broth intraperitoneally.

Group 4. Inoculum and treatment as in Group 3, but with the addition of 500 units diphtheria *antitoxin* intraperitoneally 24 hr. after the injection of *C. diphtheriae*.

Results of the therapeutic experiment are shown in Table 2.

In Group 1, the test inoculum was too small, and there were no deaths.

In Group 2, though all four pigs died, the treated pigs survived longer than the controls ($3\frac{1}{2}$ and $2\frac{1}{2}$ days as against $1\frac{1}{2}$ days in each of the two controls).

Table 2. *Fate of sixteen guinea-pigs inoculated with* Corynebacterium diphtheriae mitis *intraperitoneally or subcutaneously, and treated subsequently with or without 500 units of antitoxin, and with or without one or two intraperitoneal injections of whitmorin*

Group	1		2		3		4	
Dose of *C. diphtheriae* in millions	1·25 (I.P.)		1·25 (S.C.)		6·75 (I.P.)		6·75 (I.P.)	
Dose of whitmorin	5 c.c. + 5 c.c.		5 c.c. + 5 c.c.		10 c.c.		10 c.c.	
Interval between injections	30 min. + 24 hr.		30 min. + 24 hr.		30 min.		30 min.	
Diphtheria antitoxin	O		O		O		500 units	
Treated	S	S	$2\frac{1}{2}$d.	$3\frac{1}{2}$d.	D	S	S	S
Control	S	S	$1\frac{1}{2}$d.	$1\frac{1}{2}$d.	D	D	D	D

S, survived. D, died within $3\frac{1}{2}$ days from test injection. $1\frac{1}{2}$d.; $2\frac{1}{2}$d.; $3\frac{1}{2}$d.: died $1\frac{1}{2}$, $2\frac{1}{2}$ or $3\frac{1}{2}$ days after test injection. I.P. intraperitoneal. S.C. subcutaneous.

In Group 3, the only one survivor was in the treated group.

In Group 4, both treated animals survived, and both controls died.

DISCUSSION

It is of interest that a highly pathogenic organism such as *Pfeifferella whitmori* should produce an antibiotic resembling penicillin closely in its range of activity, dynamics of disinfection, and heat stability, and that this antibiotic should have no marked toxicity. Stanton & Fletcher (1932) have previously reported that culture filtrates of *Pf. whitmori* are non-toxic to laboratory animals. The most striking exception to the parallelism which has been drawn

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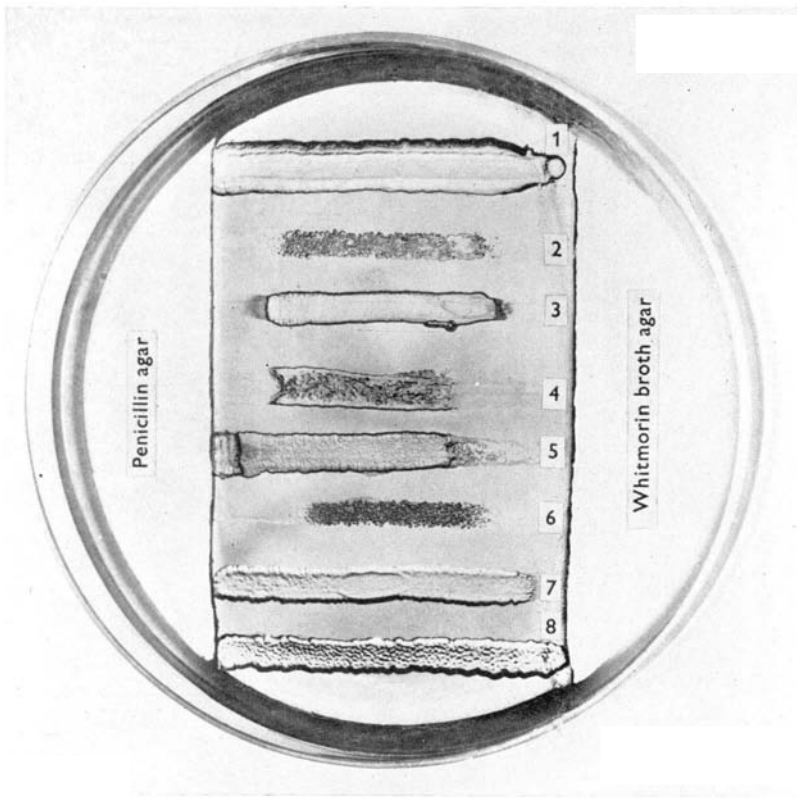

Fig. 1

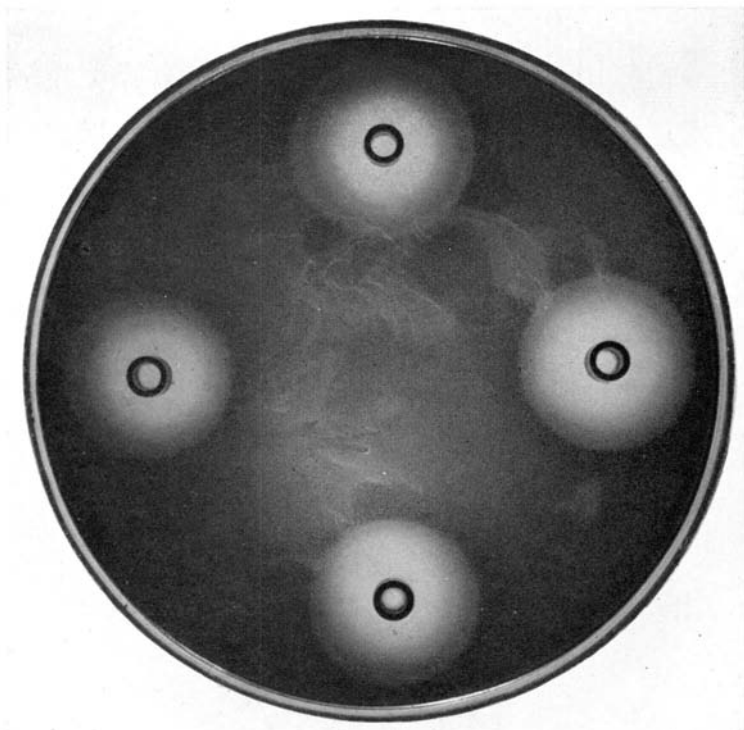

Fig. 2

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between penicillin and whitmorin is in the sensitivity of *Mycobacterium tuberculosis* to Whitmorin. There were lesser variations in the degree of sensitivity of other organisms to the two antibiotics: thus the whitmorin broth used in the therapeutic trial had a penicillin equivalent of 80 units/c.c. in bacteriostatic activity on *Corynebacterium diphtheriae mitis*, but of only 2 units/c.c. against the Oxford staphylococcus.

The true sensitivity of *M. tuberculosis* to whitmorin is difficult to evaluate owing to the slow growth of *M. tuberculosis* at 37° C., at which temperature whitmorin broth lost 55% of its potency in 72 hr.: the fact that a crude and unconcentrated preparation of whitmorin had an inhibitory titre of 1/40 against *M. tuberculosis* after 6 days' incubation at 37° C. does, however, suggest that the H 37 RV strain may be appreciably sensitive to whitmorin.

The result of the experiment in animal therapeutics was strongly suggestive in view of the crudeness of the antibiotic preparation used, though no claim is made that the figures are of high significance.

It is suggested that whitmorin might have a place in the treatment of tuberculosis, and possibly in neisserial and other infections which have acquired resistance to penicillin. The results recorded may justify further investigation.

Attention must here be drawn to the risk of working with *Pf. whitmori* in view of the extremely high mortality of melioidosis (1932), should infection occur in a laboratory worker. Little is known of the infectivity of laboratory cultures of *Pf. whitmori* in temperate climates. A search for avirulent laboratory strains of *Pf. whitmori*, or for strains of low virulence, yet capable of elaborating whitmorin, might answer the problem of laboratory risk.

SUMMARY

An antibiotic elaborated by *Pfeifferella whitmori* is described. This principle is active *in vitro* against the H 37 RV strain of *Mycobacterium tuberculosis*, but in several other respects closely resembles penicillin. It has given encouraging results in a small-scale therapeutic trial in experimental guinea-pig diphtheria.

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EXPLANATION OF PLATE 2

Fig. 1. Growth of the following organisms reading from above downwards on lysed-blood serum agar with penicillin agar 4 units/ml. on the left and whitmorin broth agar on the right. 1, *Bact. coli*; 2, *Streptococcus pyogenes*; 3, Standard Oxford *Staphylococcus*; 4, *Meningococcus*; 5, *C. diphtheriae*; 6, *Pneumococcus*; 7, *Streptococcus* Group D; 8, *S. typhi*.

Fig. 2. The photograph is of a pour plate of *C. diphtheriae mitis* in serum agar, and shows inhibition of growth round Heatley cylinders filled with whitmorin broth. Potassium tellurite had in this particular case been incorporated in the medium to accentuate the visible demarcation of the zones of inhibition.

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