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BACTERIOLOGICAL TYPING OF C. DIPHTHERIAE STRAINS RECENTLY ISOLATED IN TEHERAN

Esterabady, H. Taslimi, H. and Nategh, R. (★)

Introduction

From a total of 600 nose and throat introduction swabs examined forc. diphtheriae, 200 or 33% were positive. Cultures were carefully classified on the basis of morphological appearance and biochemical characteristics into Gravis, Mitis and Intermedius groups.

Materials and Methods

Of the 200 strains, 141 were freshly isolated at Razi Institute, 30 recieved from the Pasteur Institute in Teheran and 29 from other sources.

These were dried immediately after isolation or when recieved from laboratories.

Isolation and type differentiation:

Loeffler (1) slopes and tellurite were inoculated with nose or throat swabs taken from the patients. The presence of C. diphtheriae was determined after incubation at 37°C. for 24 hours.

A typical colony was chosen from the tellurite plate and subcultured onto a biochemical investigation and vacuum dried for preservation.

Colonial appearance on the tellurite medium

Differentiation of the three types by colonial appearance is difficult due to the absence of a standard medium.

In different laboratories preferance is expressed for various types of tellurite medium described by Mcleod, Anderson (2), Claubrg (3), Horgan and Marshall (4) Neill (5) or some other workers.

The medium used was a special type of tellurite serum agar described by Handley (6).

In additon to a peptone and Lemco agar base this medium contains a tryptic hydrolysate of caseine, sodium lactate, Lcystine, sheep serum, an extract of shee pred blood cells and 0.01% potassium tellurite.

All strains of C. diphtheria which were isolated from different loca-

(★) State Razi Institute. Hessarak. P.O. Box 656. Teheran

lities produced a characteristic transparent discoloration in the medium around each colony after 24 hours incubation. Fig 1,2,3.

The media as we have used is as follows

Agar base

Lab. Lemco	3 g
Proteose peptone (Difco)	3 g
Sodium lactate	9 ml
Pure glycerine	3 ml
Agar	6 g
Distilled water	186 ml

PH = 7.6

Autoclave at 10 lb for 15 min.

Add the following ingredients to the agar base:

Tryptic caseine	60 ml
L-cystine	6 ml
Sheep serum	15 ml
Sheep red blood cell extract	30 ml
Potassium tellurite 1%	3 ml

Distribute 20 ml volumes into each petri dish.

P.S: Tryptic caseine was prepared in the biochemistry department at Razi Institute as described by Aghdachi, Sadegh and Mirshamsy. 1961 (7).

Biochemical characteristics.

All strains were tested for sugar fermentation in Hiss serum water containing Dextrose, Maltose, Sucrose, Dextrin and Starch.

Starch fermentation is one of the most characteristic features of the Gravis type. Commercial soluble starch is hydrolysed, giving variable amounts of glucose and Dextrin, which are readily fermented by all C. diphtheriae strains.

In the present investigation very good results were obtained from laundry starch.

Hiss serum water:

Di-sodium phosphate	1 g
Peptone	5 g
Distilled water	1000 ml

Steam for 15 minutes, filter, adjust to pH 7.4, and 250 ml of horse serum,

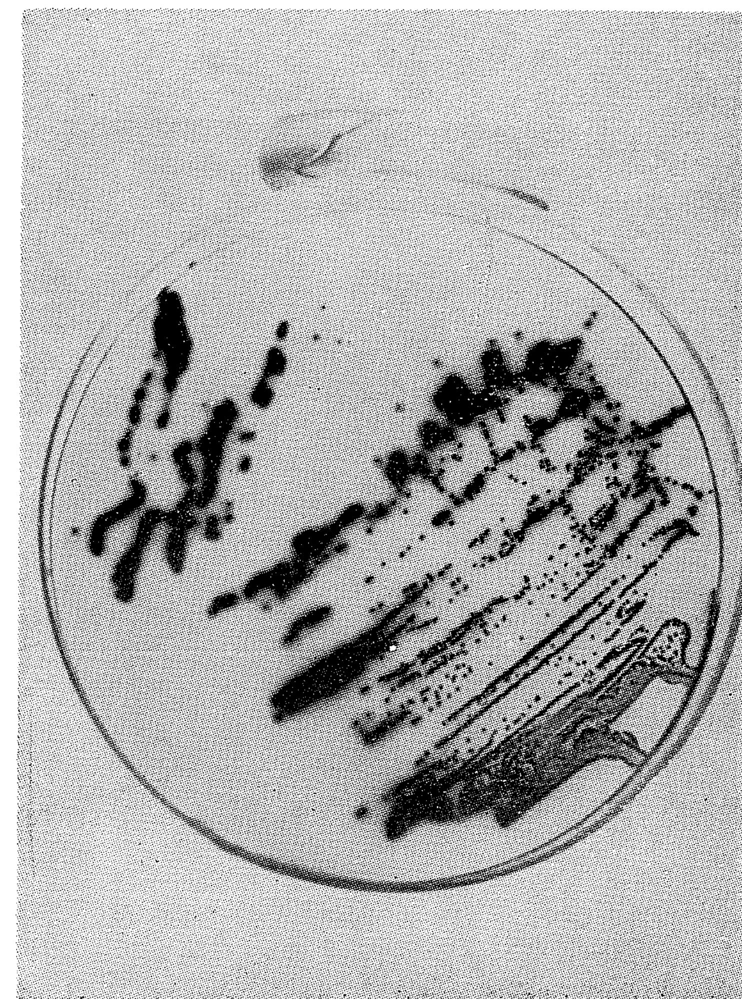

Fig 1. C. diphtheriae Gravis type on tellurite serum agar

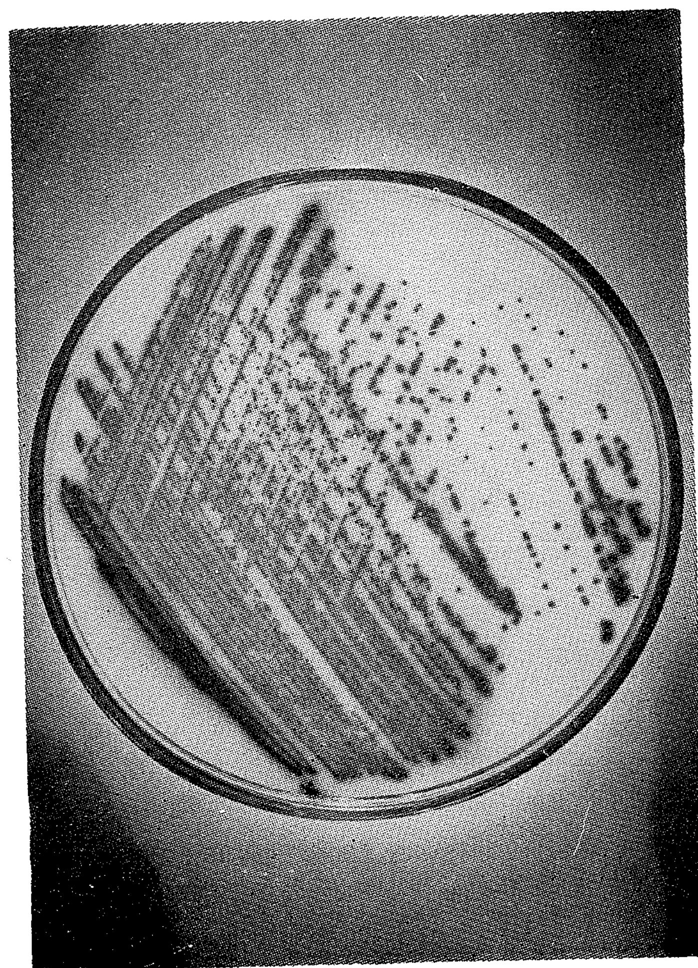

Fig 2. C. diphtheriae mitis type on tellurite serum agar.

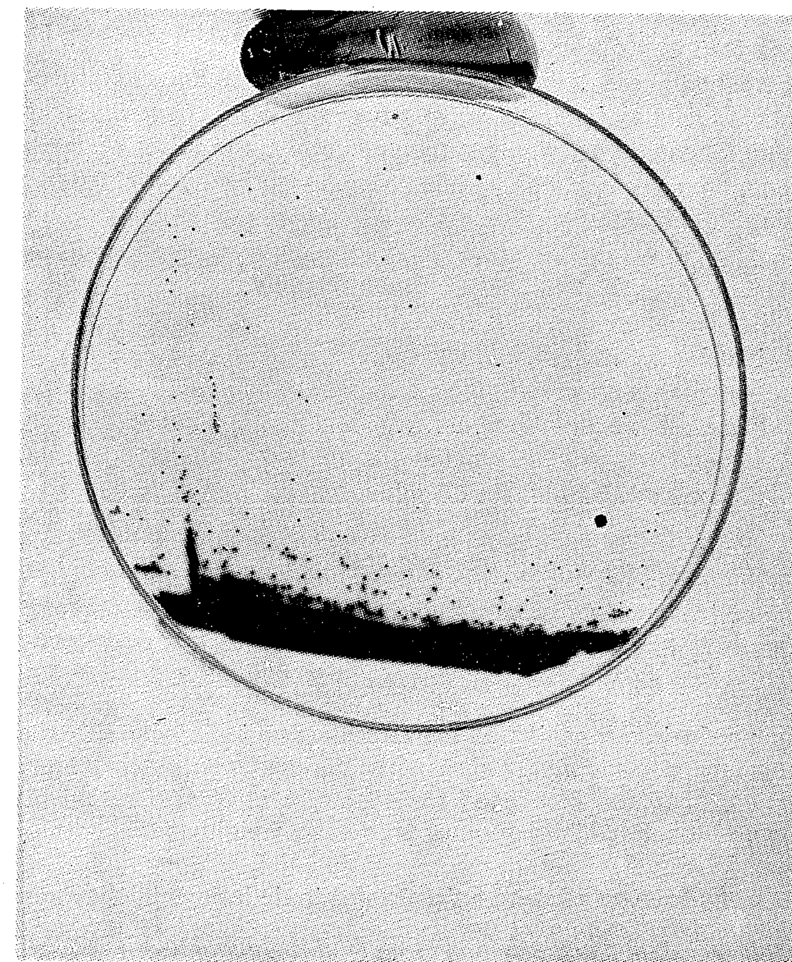

Fig 3. C. diphtheriae intermedius type on tellurite serum agar

steam for 20 minutes, add 11 ml of Brom-Tymol Blue, autoclave at 10 lb for 10 minutes, distribute 5 ml quantities in tubes and autoclave at 10 lb for 10 minutes, add to each tube sufficient amount of a sterile solution of the sugar to give a final concentration of 0.4%.

Haemolysis test:

Haemolysis test was observed in Neill's broth culture. (8)
0.5 ml of washed sheep blood added into 0.5 ml of 48 hours culture Tubes were incubated in a water bath at 37C. for one hour, after centrifugation, the degree of heamolysis was read immediately. Standing at room temprature had no effect on the result. All Gravis and Mitis types were heamolytic, but not Intermedius type.

Neill's broth culture formula:

Beef extract ....1 g
NaCl ....1 g
Proteose peptone (Difco) ....1 g
Distilled water ....100 ml
pH = 7.3
Autoclave at 10 lb. for 15 min.

Virulence test:

The study of toxigenicity of all C. diphtheriae strains was performed by in vivo and in vitro tests.

In vivo test:

The toxin producing capacity of all strains was tested by subcutaneous inoculation into guinea pigs. Tubes with 1% dextrose broth were seeded with pure cultures and incubated for 48 hours at 37°C. One ml of these cultures was inoculated into guinea pigs each weighing 250 g,the observation period being 1-3 days.
Necropsis were done on all dead animals and toxic lesions in the suprarenal glands were noted.

In vitro test

In vitro toxigenicity tests have been described by Ouchterlony (10) Parson (11) Elek (12). and others.
The filter-paper strip method introduced by Elek is widely used for in vitro virulence tests. In our experience this technique was not satisfactory. The following modification of Elek's technique was found convenient and reliable.

in this method a narrow strip of agar is cut out of the center of an agar plate and replaced with agar containing an appropriate concentration of antiserum. The pure culture to be tested was seeded in a straight line on both sides of the strip of agar containing a modified Elek's medium at right angles to it.
A combination of glycerine C.P. Tween 80 and Casamino acid (Difco) was used to replace the serum required in the in vitro test.
A volume of 0.3% solution of potassium tellurit was added to the plate to give a final concentration 0.0045% for the rapid toxigenicity test. It also has inhibitory effect on contaminating organisms. In this medium a fine precipitation appears along the streak of growth after 24 hours.
In the present series 200 cultures grown on Loeffler slants were inoculated directly onto modified Elek's medium. Precipitation of toxigenic C. diphtheriae occured after 18 hours in 186 of the cultures. Fig 4.

Modified Elek's medium:

A substitute for serum in the diphtheria in vitro toxigenicity test:
Distilled water ....100 ml
Tween 80 ....1 ml
Glycerine ....1 ml
Casamino acid (Difco) ....1 g
Add the various components to the distilled water and shake the mixture gently as each ingredient is added.
Solution occures more rapidly if the mixture is warmed in the water bath (50 to 55°C.) for a few minutes. Although this solution is acid it has not been necessary to adjust the pH.
Sterilize the additive by autoclaving at 15 lb pressure (121C.) for 12 min. The solution appears turbid when it is removed from the autoclave but clears when it is cool. It may be stored in the refrigerator for at least 4 weeks.

Modified agar base:

Proteose peptone ....2 g
Maltose ....0.3 g
Lactic acid ....0.07 ml
NaCl ....0.5 g
Agar ....2 g
Distilled water ....100 ml
pH = 7.8
Sterilize at 15 lb. for 15 min.

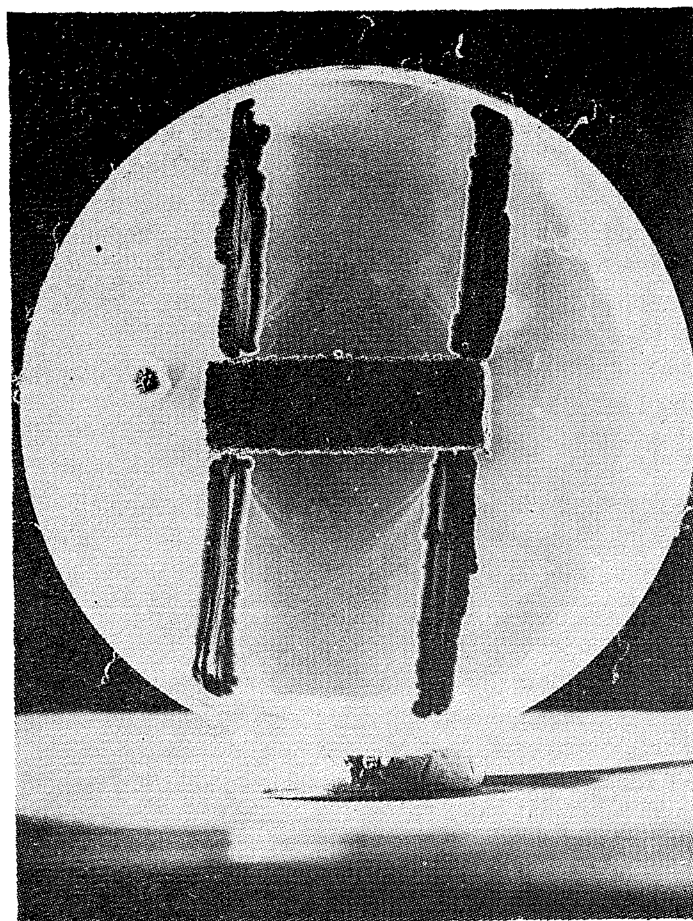

Fig 4. In vitro virulence test on modified Elek's medium

1.5 ml of a 0.3% solution of potassium tellurite is added to the plate for the rapid toxigenicity test.
The additive is used in the plate in the same quantity,2 ml,as the serum for which it is substituted.

Sensitivity test:

In this study a plate method for the determination of sensitivity was employed and nutrient broth at pH 7.3 was used as the culture medium. Elek's medium was used in the preparation of antibiotic-containing plates. Commercially prepared antibiotic discs were used (Bacto Unidiscs). The discs having the greater quantity of antibiotic were used routinely in this test. The surface of Elek's medium is flooded with a few drops of young culture.
Excess fluid is pipetted off and the plate allowed to dry.
Discs are placed on it and the culture incubated overnight.
After incubation the diameter of the zone of inhibition surrounding each disc is measured. (Fig. 5)
The following antibiotics were used in the tests with all strains:
Aureomycin 30 mcg. Chloromycetin 30 mcg. Erythromycin 15 mcg. Penicillin 10 units. Stereptomycin 10 mcg. Tetracycline 30 mcg. Sulfadiazin 300 mcg. Terramycin 30 mcg. These antibiotics can be arranged according to their activity against all strains of C. diphtheriae as follows:

Erythromycin	11—17 mm
Chloromycetin	10—16 mm
Tetracycline	7—10 mm
Penicillin	6—10 mm
Terramycin	7—9 mm
Aureomycin	5—8 mm
Sulfadiazin	2—5 mm

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Summary

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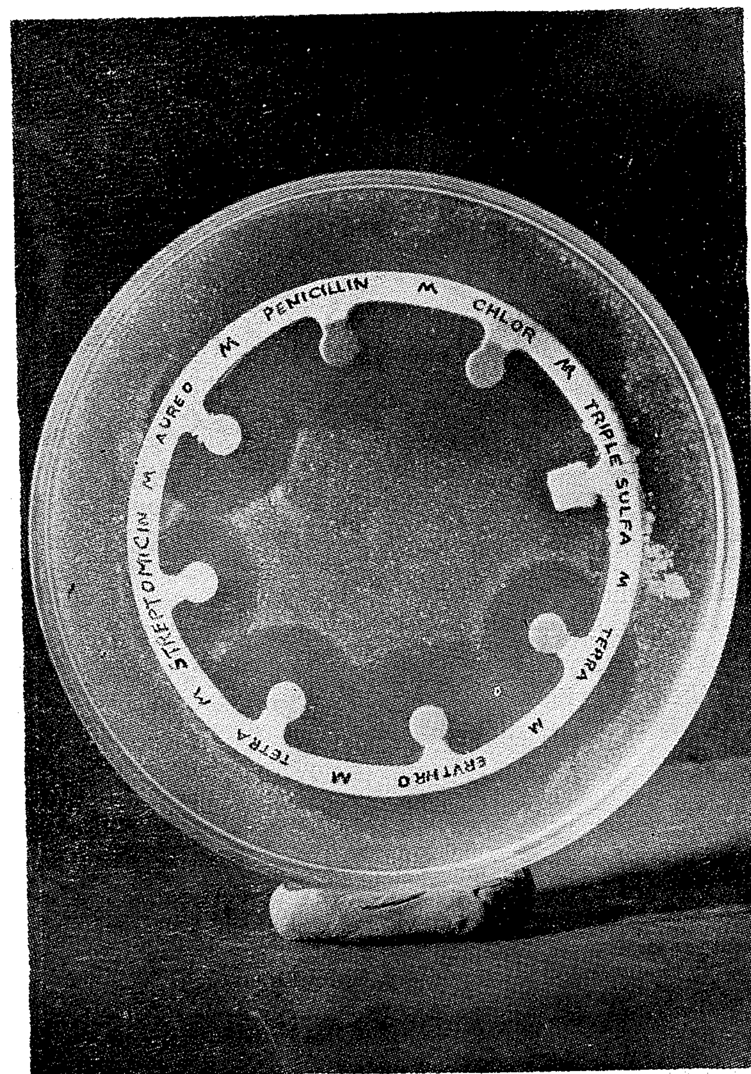

Fig 5. Antibiotic sesitivity test on Modified Elek's medium

A special tellurite serum agar was used for colonial appearance.
Neill's broth culture was employed for haemolytic tests.
The virulence of each culture was examined in laboratory animals by the agar gel precipitation method of Elek.
From 200 cultures tested, 138 or 69% were gravis, 5 or 2.5% were intermedius, and 57 or 28% were mitis.
Three strains of gravis type and one strain of mitis type were avirulent.

Sommaire

Un total de 200 souches de bacille diphtherique isolées a Teheran et environs sont classifiees sur la base de leur caractéres morphologiques et biochimiques en groupes de gravis (138=69%), intermedius (5=2.5%) et mitis (57=28%). Seul trois souches de type gravis et une souche de mitis etaient avirulent.

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