INVESTIGATION INTO THE GROUPS AND VIRULENCE OF CORYNEBACTERIUM DIPHTHERIAE STRAINS ISOLATED IN SINGAPORE

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PREFACE

To the best of our knowledge there is no previous report on the groups of Corynebacterium diphtheriae present in Singapore. Even unreported data of investigation are unavailable. The information that is used is largely based on research done in other countries. Bhagwan Singh (1955) examined 141 strains of C. diphtheriae isolated from 1790 throat swabs in the Federation of Malaya. Although in his report he mentioned the cultural and biochemical methods used by Hewitt (1947) for the classification of these organisms, he did not state if his strains were also examined by the same methods. His cultures were sent to Australia and serologically typed by Ferris, who found 98% mitis, 2% gravis and no strains of intermedius. Das and Ghoshal (1951), working in Calcutta, examined 115 cultures and all were described as mitis group.

In Singapore it is generally believed, that only mitis strains are present, whereas other groups are rare. The problem of virulence has not been examined either. Facts with regard to virulence were not systematically studied and could not, therefore, provide a clear picture.

The object of this paper is to revise present knowledge on C. diphtheriae in Singapore and to add information on their incidence and virulence.

MATERIALS AND METHODS

Morphological, cultural and biochemical Characteristics.

Throat, nose, ear and sore swabs were obtained from hospitalised and patients not in hospital. Throat swabs from healthy contacts were also included. In all 7519 specimens were collected in the period of July 1961 — January 1962. On arrival in the laboratory the swabs were immediately inoculated onto Loefflers' and tellurite media. Of the 7519 specimens 694 (9.2%) contained diphtheria. For the sake of accuracy only 378 will be considered here. These cultures were selected at random and subjected to careful identification. Prior to that each strain was transferred through B.C.T. medium (Monekton's Blood-Copper-sulphate-tellurite broth, cited by Mackie & McCartney, 1953) and purity checked by plating out on Loefflers' plates. Our classification into groups was based on colony morphology, growth in nutrient broth, fermentation of dextrose, maltose, dextrin, starch and on the ability to haemolyse human or rabbit red blood cells. The haemolysis was tested on blood plates, which gave results comparable to those obtained by Hewitt (1947) with 3% rabbit blood in papain digest broth. Our criterion for gravis strains was, that they appeared on tellurite plates as typical daisy-headed colonies, were not evenly emulsified in saline, grew in granular deposit in broth and fermented dextrose, maltose, dextrin and starch. Both human and rabbit red cells were moderately haemolysed. Involution forms were more marked if the cultures were incubated for few more days at room temperature. Our intermedius strains were similar to gravis strains in colony morphology and involution formation, but were more easily emulsified. They did not hydrolyse starch and did not haemolyse red cells. Broth culture was turbid with little deposit. Colonies of mitis strains were smooth, glistening and dark-grey to black on tellurite plates. They did not attain the same colony size as gravis, appeared buttery in consistency and were easily emulsifiable. Involution forms were not formed on prolonged incubation at room temperature. Growth in broth was turbid with occasional slight deposit, that disappeared after gentle shaking. Slight haemolysis was always present. The fermentation reactions were same as with intermedius strains.

Animal virulence test

Hewitt (1947) used lethal effect in guinea-pigs as a test for virulence and intradermal injections of horse-flesh digest culture filtrate as a toxigenicity test. At first we used an overnight broth culture and inoculated 0.1-0.2 intradermally. Later, we found that it was more convenient to use suspensions of blood-agar culture in nutrient broth. Typical character of growth could again be confirmed and contamination could be detected. For the intradermal test rabbit or guinea-pig skin was shawed a few hours before injection and a fine needle was used to prevent possible escape of material. In rabbits, as many as 15 injections and 9 injections in guinea-pigs, did not interfere with reading the results. Four to five hours after injection of diphtheria organisms

rabbits were given 400-500 units and guinea-pigs 100-150 units of diphtheria antitoxin intraperitoneally. Normally, Burroughs-Wellcome refined (3000 units/c.c.) antitoxin was used, otherwise Sclavo antitoxin (of 2000 units/c.c.) was given. Results were best recorded after four days when the positive strains produced well developed necrotic areas.

In-vitro toxigenicity test

A slight modification of Elek's (1949) agar-gel diffusion method was used for the in-vitro toxigenicity test in preference to Ouchterlony's method (1948). In the test, Oxoid ionagar No. 2 was found to be as good as Special Nobel agar, Difco. Burroughs-Wellcome or Evans horse serum, inactivated for half an hour at 56°C on three successive days was added to the molten agar in a 20% concentration. The best precipitation lines were obtained with Burrows-Wellcome diphtheria antitoxin and it was, therefore, used throughout our investigation. Sterile 15 mm wide filter paper strips were saturated with a solution that contained 1000 units/c.c. of antitoxin and placed on the bottom of a sterile Petri dish. It was found that, if molten agar was poured onto the filter paper strips, these did not float or move, as often happens when they are dipped into the liquid medium. The plates were dried for a few hours in an incubator and within six hours of preparation they were inoculated from a twenty four hour blood agar slant culture. After one day preliminary incubation for ensurance of rich growth and check for contamination, the plates were reincubated for another 2-3 days in glass-jars wrapped in nylon. The main precipitation lines appeared in 1-2 days, but were recorded after 3-5 days when their intensity had increased. Many strains produced a number of secondary lines, which appeared later, but for the sake of simplicity, these have been ommitted.

RESULTS AND CONCLUSIONS

Table 1 shows that approximately one third of the specimens were from contacts. Once a contact is discovered he is immediately hospitalised and treated irrespective of clinical symptoms. Repeat specimens, therefore, include diseased as well as apparently healthy patients. As can be seen the percentage of positive cultures amongst contacts is relatively high (2.5%). In Singapore 9.2% positive cultures were obtained as compared with 8.2% in the Federation of Malaya.

As shown in Table 2, gravis and intermedius strains are well represented in Singapore. Gravis comprises 15%, intermedius 20% and mitis the majority, 65%. As far as virulence is concerned there is a slight difference among the groups. Only half (52%) of the local strains of gravis, which is generally regarded in western countries as the most virulent, are virulent, whereas three quarters (74%) of the local strains of mitis, regarded as least pathogenic in the west, proved

TABLE 1

Diphtheria cultures isolated from specimens of hospitalised and contact patients.

Specimens from		Total number of specimens	Positive cultures obtained from		
Hospital patients (includes repetitions)	Contacts		Hospital patients (includes repetitions)	Contacts	Total isolates
4998	2521	7519	630 (12%)	64 (2,5%)	694 (9,2%)

TABLE 2

The distribution of gravis, intermedius and mitis strains among 378 diphtheria cultures and their in-vivo and in-vitro virulence.

Diphtheria group	Total number	Number virulent
Gravis	59 (15%)	31 (52%)
Intermedius	75 (20%)	48 (64%)
Mitis	244 (65%)	182 (74%)

to be virulent. The intermedius group occupies an intermediate position.

Table 3 illustrates the distribution of diphtheria groups in throat, nose, ear and sore specimens with respect to diseased persons and contacts. Repeat cultures are entered separately. Attention should be drawn to the relatively high percentage of virulent strains found among contacts or, perhaps, carriers. With the exception of the intermedius strains (2 out of 9), the gravis (4 out of 6) and the mitis strains (16 out of 22) recovered from contacts were as virulent as those from diseased persons. The findings in cultures of specimens from nose should also be emphasized. Contrary to findings in western countries our cultures of specimens from the nares did not differ from cultures of throat material with respect to virulence. Although the number of strains examined is not high, it does show, that all of the strains isolated from nose or throat are of approximately equal virulence.

TABLE 3

The incidence of gravis, intermedius and mitis strains in various specimens from various patients and result of their virulence test.

Swab specimen taken from	Kind of Patient	Total No. tested	Cultures virul.	Cultures non vir.	Total tests	Total virul	Percent virul.
Gravis							
Throat	Diseased Repetitions Contacts	27 14 6	15 5 4	12 9 2	47	24	51%
Nose	Diseased Repetitions	8 2	3 2	5 —	10	5	50%
Ear	Diseased Repetitions	2 —	2 —	— —	2	2	
Sore	Diseased Repetitions	— —	— —	— —	—	—	
Intermedius							
Throat	Diseased Repetitions Contacts	40 9 9	30 5 2	10 4 7	58	37	64%
Nose	Diseased Repetitions	12 —	8 —	4 —	12	8	75%
Ear	Diseased Repetitions	2 —	1 —	1 —	2	1	
Sore	Diseased Repetitions	3 —	2 —	1 —	3	2	
Mitis							
Throat	Diseased Repetitions Contacts	159 27 22	124 18 16	35 9 6	208	158	71%
Nose	Diseased Repetitions	22 5	17 1	5 4	27	18	66%
Ear	Diseased Repetitions	4 —	3 —	1 —	4	3	
Sore	Diseased Repetitions	3 2	3 —	— 2	5	3	

Corynebacterium cultures involved in lethal cases of diphtheria

In order to gain information on the strains incriminated in the death cases we examined a dozen diphtheria fatal cases that occurred in the period of our investigation. In 2/3 of them strains belonging to mitis group were involved and in one third intermedius group. Gravis was not included among these, but this point can be elucidated only by examining a large number of death cases.

In-vivo virulent in-vitro avirulent strains

At the beginning of our investigation we examined the virulence of diphtheria cultures by the animal intradermal method. We soon introduced Elek's agar-gel-diffusion technique and both tests were conducted simultaneously on each culture. To date only two strains disagree in their results. The two strains produced necrosis in rabbit or guinea-pig skin, but failed to produce precipitation lines in the gel-diffusion plates. 100 units of antitoxin given to a guinea-pig or 500 units to a rabbit, four hours after injection, did not inhibit the skin, reaction, but if 1000 and 5000 units respectively were given before the intradermal inoculation, the skin reaction did not develop. Similar results were reported by Hartmann (1959).

DISCUSSION

As already mentioned our aim was to investigate the incidence and virulence of diphtheria groups in Singapore. For this we chose the biochemical classification. Serological classification will be considered separately in a later communication. According to Ferris (1951) the fermentation of starch by strains of gravis is a conclusive criterion irrespective of colony morphology. Haemolytic properties were not considered significant by him, whereas Hewitt took it as a diagnostic aid. We also considered starch fermentation specific for gravis and believe that among our strains no others are included. Moreover, the number of strains of gravis may be even higher since loss of the property of starch fermentation can lead to intermedius or mitis group (Hewitt, 1947).

The degree of virulence is generally related to the different diphtheria groups. In the western countries gravis is known to be more common, more virulent and more fatal. The situation is reported to be different in this region, where almost only mitis strains are prevalent (Das 1951, Bhagwan Singh 1955). Singapore is between the above extremes. The percentage of virulent mitis strains is a little higher, 74% as compared with 52% of gravis. In a study of 274 strains by Hewitt (1948) 91% of his 71 gravis, all his 21 intermedius and 80% of his 180 mitis strains were virulent. He found no evidence of gravis to be more rapidly lethal than mitis. On examining a dozen fatal cases of diphtheria we found two thirds due to mitis, one third due to intermedius and not a single case due to gravis.

The examination of contact cases revealed interesting information. 2.5% of the contacts were positive for diphtheria and contained strains of all the groups. These contacts (or carriers) constitute a hazard to the public, since they harbour diphtheria organisms of the same virulence as those carried by diseased patients. Although more than one strain of diphtheria can be isolated from a single swab (Hewitt 1947), only one kind of diphtheria was recovered from all of ours: swabs from different sources but of same patient yielded the same strain.

The two mentioned strains, which were virulent in animals but not toxigenic in plates, should be regarded as virulent and toxic. The antigenic structure of their toxins is probably not identical with strains employed in manufacture of the diphtheria antitoxin used in our agar-gel-diffusion plates. This fact supports the opinion of Green (1951) who found, during the Japanese occupation of Malaya, that the imported commercial antitoxin was less effective than the crude antitoxin he prepared himself from local strains in goats.

SUMMARY

1. 7519 throat, nose, ear and sore swabs were examined for diphtheria, of these 694 or 9.2% yielded positive cultures.

2. Of the above cultures 378 were selected at random and carefully classified on the basis of morphological, cultural and biochemical characteristics into gravis, intermedius and mitis groups. Of the above 59 or 15% were found to be gravis, 75 or 20% were found to be intermedius and 244 or 65% were found to be mitis.

3. The virulence of the cultures was examined in laboratory animals and by the agar-gel-precipitation method of Elek; 51% of gravis, 64% of intermedius and 74% of mitis strains were found to be virulent.

4. Cultures obtained from nose swabs had about same percentage of virulent strains as cultures from throat swabs.

5. Diphtheria organisms were found in 2.5% of the throat swabs from 2521 apparently healthy contact patients. In these all three groups were found and the virulence of the strains was same as from diseased patients.

6. Methods of classification and significance of findings in swabs from contact patients were discussed.

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