

Screening tests for pathogenic corynebacteria

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Abstract

Aim: To provide simple tests that would help in the identification of corynebacteria that produce diphtheria toxin.

Methods: A collection of 99 freshly isolated corynebacteria was assembled and the cultures identified by conventional tests confirmed by an identification kit. Modifications were made to procedures for preparation of the culture medium for the Elek test and to the test for detection of pyrazinamidase (pyrazine carboxylamidase) activity. These two together with an indicator medium for cystinase activity were applied to the collection of organisms.

Results: Cystinase was detected in all 61 members of the toxigenic species and none produced pyrazinamidase. In contrast, all but two of the 38 representatives of non-toxicogenic species yielded pyrazinamidase and none formed cystinase. Of the 61 cystinase producing cultures (which were also pyrazinamidase negative), 21 gave a positive Elek test with the modified culture medium. A total of 30 of these 61 were tested for toxigenicity in guinea pigs and the results of the animal and plate tests concurred. At least seven cultures could have been reported as non-toxicogenic if Elek tests based on media prepared in the conventional way had been the only test available.

Conclusion: The three procedures described go some way towards meeting the needs of diagnostic laboratories for efficient procedures for distinguishing pathogenic corynebacteria.

Corynebacterium diphtheriae and the true diphtheroids, *C. ulcerans* and *C. pseudotuberculosis* (*C. ovis*), have some biochemical characteris-

tics in common and all may produce the diphtheria toxin.¹ *C. diphtheriae*, as well as causing diphtheria,² can be present in skin ulcers,³ and non-toxicogenic strains have been recovered from sore throat⁴ or patients with endocarditis.⁵ *C. ulcerans* has been occasionally isolated from bovine mastitis in the United Kingdom⁶ and rarely from diphtheria in man.⁷ *C. pseudotuberculosis*, a cause of caseous lymphadenitis in sheep and goats, has been found recently in the United Kingdom by veterinarians,⁸ and elsewhere it has caused axillary or inguinal lymphadenitis in handlers of infected animals.⁹

Attention has been drawn to the value of the routine use of media that are selective for *C. diphtheriae*.⁴ Simple screening tests are needed to determine whether an isolate belongs to a potentially toxicogenic species and secondly improvements in the methods of detecting toxigenicity are also needed.¹⁰ This paper supplements a description of the bacteriological diagnosis of diphtheria² and deals with three procedures that we have found useful in the initial handling of suspected pathogenic corynebacteria.

Methods

A total of 99 fresh isolates were used and are listed in the table. They were, with the exception of the representatives of *C. pseudotuberculosis*, from human clinical sources and had been referred to the Diphtheria Reference Laboratory for examination. The species identification was based on the results of both standard biochemical tests² and a commercial kit ("API Coryne"; API-bioMérieux, Basingstoke).

Control strains for the pyrazinamidase (pyrazine carboxylamidase) test—namely, *C. ulcerans* (NCTC 12077) and *C. xerosis* (NCTC 12078), have been deposited with the National Collection of Type Cultures. The three standard control strains of *C. diphtheriae* (NCTC 10648, NCTC 3984, NCTC 10356) were used in all Elek tests.²

PYRAZINAMIDASE ACTIVITY

Solutions of pyrazinamide (2 mg/ml; Sigma) were prepared in deionised water, sterilised by filtration through membrane filters (0.45 µm), bottled and stored frozen. Ammonium ferrous sulphate hexahydrate solutions (20% w/v) were similarly stored frozen but not sterilised.

For use, 0.5 ml of thawed pyrazinamide solution was added to each of three sterile capped plastic tubes for the controls and one tube for each test strain. Tubes were inoculated

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Accepted for publication
4 July 1991

Distribution of positive results obtained with three tests among 99 isolates referred to the Diphtheria Reference Laboratory

| Organism | No | Pyrazinamidase | Cystinase | Elek test | Guinea pig results* |
|--|----|----------------|-----------|-----------|---------------------|
| <i>C. diphtheriae</i> | 47 | 0 | 47 | 19 | 9/24 |
| <i>C. diphtheriae</i> var. <i>belfanti</i> | 9 | 0 | 9 | 0 | 0/3 |
| <i>C. ulcerans</i> | 2 | 0 | 2 | 2 | 2/2 |
| <i>C. pseudotuberculosis</i> | 3 | 0 | 3 | 0 | 0/1† |
| <i>C. pseudodiphtheriticum</i> | 3 | 1 | 0 | 0 | NT‡ |
| <i>C. xerosis</i> | 6 | 6 | 0 | 0 | NT |
| <i>C. jeikeium</i> | 6 | 6 | 0 | 0 | NT |
| Corynebacteria, group D2 | 6 | 6 | 0 | 0 | NT |
| Corynebacteria, group G1 | 1 | 1 | 0 | 0 | NT |
| <i>C. minutissimum</i> | 1 | 1 | 0 | 0 | NT |
| Other corynebacteria | 15 | 15 | 0 | 0 | 0/1 |

* No positive/No infected.

† 'ovis' toxin produced causing localised abscess.

‡ not tested.

from overnight cultures on blood agar to give a density at least equal to that of the MacFarland No 2 tube and were incubated overnight. After incubation one or two drops of ferrous sulphate solution were added to each tube and if an orange-red colour developed immediately this was taken as a positive test for pyrazinamidase activity. An uninoculated tube of pyrazinamide (colourless after the addition of ferrous sulphate) served as a negative control and 0.5 ml of a solution of pyrazine carboxylic acid (1 mg/ml; Aldrich) as a positive control. Positive control cultures were *C xerosis* (NCTC 12078); negative were *C ulcerans* (NCTC 12077).

CYSTINASE ACTIVITY

Enzyme activity was detected in modified Tinsdale medium.

Base: Bacto-Tinsdale base (Difco), the components of the Difco medium or Tinsdale agar base (Oxoid) were all satisfactory in our hands.

Supplements: (ml/100 ml base) were added in the following order:

- (a) 10 ml of horse serum.
- (b) 0.11 ml of cystine solution (20% w/v) was added drop by drop, mixing during addition. Cystine is incorporated in the Oxoid base and further addition is contraindicated if that preparation is used. The solution was prepared by dissolving 20 g of L-cystine in 100 ml of 2M hydrochloric acid. The solution is self-sterilising and can be stored at room temperature in the dark.
- (c) 2.5 ml of potassium tellurite solution (1% w/v) which was prepared by dissolving 1 g of potassium tellurite in 100 ml of sterile deionised water. It is self-sterilising and is stable for up to six months if stored in the dark at room temperature.
- (d) 1.5 ml of a solution of sodium thiosulphate (2.5% w/v). A convenient volume of a solution of the anhydrous salt was prepared and sterilised by filtration always just before use.

Plates were poured and stored in the refrigerator for up to two weeks.

Inoculation: a sweep from a fresh culture was spread across most of the surface of an agar plate to give individual colonies. The remainder was then inoculated with a cystinase producing control organism: either *C ulcerans* (NCTC 12077) or *C diphtheriae* (NCTC 10648). The cultures were incubated for two days at 37°C in air. Cystinase producing cultures formed black colonies surrounded by a brown halo, usually after overnight incubation, but stock cultures of *C diphtheriae* var. *intermedius* have given a delayed reaction.

ELEK TEST

The base was prepared in two parts.

- (a) 20 g of a suitable batch of Proteose peptone No 2 (Difco) was added to 500 ml of deionised water, dissolved, then 3.25 ml of a 40% (w/v) solution of sodium hydroxide was added, stirring constantly. The solution was then heated to boiling in a steamer, cooled, and filtered through glass fibre paper (grade GF/F; Whatman) to remove the precipitate. Lactic acid (0.7 ml) (90% solution; Analar) was added, followed by 3 g maltose, and finally the reaction

was adjusted to pH 7.8 with 1M hydrochloric acid (Analar).

- (b) 5 g sodium chloride (Analar) and 10 g of Agar No 1 (Lab M, code MC2) were added to 500 ml deionised water, mixed, allowed to stand cold, and then steamed to dissolve the agar. After cooling to 50°C, the solution was brought to pH 7.8 with 1M sodium hydroxide (Analar).

Parts a and b were mixed together, distributed in 15 ml amounts in McCartney bottles, and autoclaved for 10 minutes at 116°C. The prepared base had a shelf life of three months.

For use, 3 ml of newborn calf serum was added to the molten base at 45°C and the plates poured. Fetal calf serum was not satisfactory. The test was then performed as described by Brooks and Joynson.²

Over one 21 month period 19 isolates of potentially toxigenic species were received and Elek tests were carried out in triplicate using the medium described above and also bases that had been prepared in the conventional way¹¹ in two laboratories X and Y.

Results

The distribution of the three properties in 99 cultures is given in the table. All 61 cultures belonging to one or other of the three species of direct public health importance—namely, *C diphtheriae*, *C ulcerans*, and *C pseudotuberculosis*, produced cystinase but not pyrazinamidase. The other 38 cultures did not produce cystinase, and all but two, both cultures of *C pseudodiphtheriticum*, produced pyrazinamidase. The API Coryne kit includes a test for pyrazinamidase and the results with the kit and the method described here were entirely consistent.

A positive Elek test was obtained, using the medium given here, with 21 of the 61 cystinase producing pyrazinamidase negative cultures. Validation was provided by use of the subcutaneous test for toxigenicity in paired guinea pigs (one of which was protected with diphtheria antitoxin) and comparison with Elek tests in which media prepared by the conventional method was used. Altogether, 31 cultures were injected into guinea pigs. Of these, one strain that formed pyrazinamidase was avirulent in guinea pigs and gave a negative Elek test. One strain of *C pseudotuberculosis* was tested in guinea pigs. Abscesses formed at the site of inoculation in both test and control animals and this was taken as evidence that the "ovis" toxin, a phospholipase, was being produced. There was no evidence of the production of diphtheria toxin by this culture in either the Elek test or in animals. Strains from 29 others, all pyrazinamidase negative cultures, were tested in guinea pigs. The results of the guinea pig tests and the Elek tests were consistent. Diphtheria toxin was detected in nine cultures of *C diphtheriae* and two of *C ulcerans* but in none of 15 other isolates of *C diphtheriae* nor in any of the three representatives of *C diphtheriae* var. *bel-fanti*.

Among the 19 isolates on which Elek tests

were carried out in triplicate, there were seven discrepancies. With medium from laboratory X the Elek test was negative with one culture of *C ulcerans* that was toxigenic in the guinea pig. Diphtheria toxin was not detected in Elek tests performed on six cultures of *C diphtheriae* using base prepared in laboratory Y. These six cultures were toxigenic in guinea pigs.

Discussion

The absence of pyrazinamidase in *C diphtheriae*, *C ulcerans*, and *C pseudotuberculosis*, but its presence in representatives of other species of corynebacteria, was described a decade ago.¹² The test method applied then was one used for detecting pyrazinamidase activity in mycobacteria and in our hands could not be made to work with corynebacteria. To date the absence of any description of the presence of pyrazinamidase in the three toxin producing species suggests that it is a useful screening test for these organisms. Our finding of two strains of *C pseudodiphtheriticum* that did not produce the enzyme indicates, however, that it should not be the only screening test used.

Cystinase is formed by strains of potentially toxigenic species. We have used agar plates containing modified Tinsdale medium to detect activity. Alternatively, tubes of the same composition, or of modified Pisu's medium,¹³ could have been inoculated by stabbing. Stab inoculation of tubes may give a quicker result but our justification for using surface growth is the possibility of detecting cystinase producing colonies in mixed culture. This was possible with artificial mixtures (data not shown).

Limitation of iron is an essential factor in the expression of the gene for diphtheria toxin. For this reason an agar powder with a low ash content is specified here for the Elek test medium. Removal of precipitated phosphates, the importance of which was emphasised by Elek,¹¹ is carried out with glass fibre filters before the addition of lactic acid, not after-

wards. In the modified procedure precipitates were removed at pH 11 instead of pH 9.5. After these changes it has not been difficult to detect diphtheria toxin, even among weakly toxigenic strains such as NCTC 3984.

Full identification of isolates, together with confirmation of toxigenicity, requires further testing, and some of these procedures are best carried out in reference laboratories. It is essential, however, to have reliable screening tests in peripheral laboratories and this study was started for that reason.

We are grateful to Dr B D Cookson and also Mr R Brooks, Ms Gillian Hallas and Mr M Mead for helpful discussions and advice.

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