SHORT ARTICLES

GROUPING OF β -HAEMOLYTIC STREPTOCOCCI BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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IDENTIFICATION of β -haemolytic streptococci (BHS) in the diagnostic laboratory has depended traditionally on the use of presumptive tests in the first instance (Wilkinson, 1979). Definitive group identification rests on extraction of group-specific carbohydrate antigens and their reaction with specific antisera in capillary tubes or agar gel; counterimmunoelectrophoresis may also be used (Hill *et al.*, 1975). Other methods of identification include slide agglutination tests (Christensen *et al.*, 1973) and fluorescence staining (Moody, Ellis and Updyke, 1958).

The present study was undertaken to determine whether an enzyme-linked immunosorbent assay (ELISA) could be adapted to provide a reliable system for grouping BHS. Although this type of assay has been employed for detection of protein antigens and antibodies (Engvall and Perlmann, 1972; Russell, Facklam and Edwards, 1976), many workers have been unable to apply it to polysaccharide antigens, which have an overall negative charge that interferes with adsorption to polystyrene surfaces (Dr B. M. Gray, Dept of Pediatrics and Microbiology, University of Alabama in Birmingham, USA). In this paper we report that this difficulty can be overcome by adsorbing whole cells to polystyrene plates. Results of a trial of the ELISA procedure compared with a double-diffusion method in agar gel are presented.

MATERIALS AND METHODS

Streptococci were isolated from patients attending the Royal Infirmary, Edinburgh, or were stock strains held in the laboratory. The 100 strains tested were: 20 group A, 35 group B, 15 group C, 15 group D, and 15 group G. Initially, strains were identified by the following presumptive tests: gram-stained film, haemolysis on blood agar (Brown, 1919), bacitracin sensitivity for group A (Maxted, 1953), the CAMP test for group B (Christie, Atkins and Munch-Petersen, 1944) and the bile-aesculin test for group D (Facklam and Moody, 1970).

Preparation of extracts. The group-specific carbohydrates were extracted as described by Lancefield (1933). Each strain was grown in 50 ml of Todd-Hewitt broth for 18 h at 37°C and the culture was centrifuged at 1000 *g* for 15 min. The packed cells were extracted with 0.4 ml of 0.067M HCl for 10 min. at 100°C, cooled, and neutralised with 0.5M NaOH. After centrifugation, the supernate was stored at 4°C.

Gel-diffusion test. Double diffusion in agar gel was performed by a modification of the Ouchterlony method (Freimer, 1963).

Antisera. Grouping sera for groups A, C, D and G were obtained from Wellcome Reagents Ltd, Hither Green, London SE13 6TL, and used for the ELISA and for the agar double-diffusion tests. Group-B antiserum was raised in rabbits in our own laboratory. Strain O90R (*Streptococcus* Reference Laboratory, Colindale, London NW9 5HT) was grown in 250 ml of

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Todd-Hewitt broth for 18 h at 37°C, washed twice in 0.9% (w/v) NaCl, killed by suspending in 2% (v/v) formalin for 3 h, and then resuspended in 25 ml of 0.9% NaCl.

An initial intravenous dose of 0.5 ml of vaccine was given and then 1 ml of vaccine was given intravenously twice weekly for 1 month; test bleeds then showed that there had been a strong homologous response by each animal. The rabbits were exsanguinated and the sera, divided into 5-ml amounts, were stored at -20°C until use. Merthiolate (1 in 10 000) was used as a preservative.

Cross-reacting antibodies were absorbed from each of the grouping sera by adding sequentially 10 ml of a 1 in 10 dilution of serum in PBS (0.05M sodium phosphate buffer containing 0.15M sodium chloride, pH 7.4) to packed PBS-washed bacteria from 100 ml of overnight culture of the prototypes representing the heterologous groups (group A strain NCTC8198, group B NCTC9993, group C NCTC8543, group D Colindale strain and group G NCTC9603) and incubating for 30 min. at room temperature.

Preparation of ELISA plates. Each strain was grown in 5 ml of Todd-Hewitt broth incubated aerobically overnight at 37°C. After the cells had been washed once in saline, 0.5 ml of 0.05M sodium carbonate buffer at pH 9.6, containing 0.02% (w/v) of sodium azide (NaN_3) was added, and 50 μl of this suspension was dispensed into wells of a disposable flat-bottomed polystyrene microtitration plate (Sterilin Ltd, 43-45 Broad Street, Teddington, Middlesex). The plate was left open and placed in a drying cabinet at 60°C for 1 h to allow cells to adhere to the polystyrene surface.

The ELISA technique was similar to that described by Voller, Bidwell and Bartlett (1976). Each well was washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBST), and shaken dry at the end of each wash; 50 μl of a 1 in 400 dilution of the test serum in PBST with 0.02% (w/v) NaN_3 was then dispensed into the wells and allowed to incubate at 37°C for 1 h. To prevent evaporation, each plate was sealed in aluminium foil. Washing with PBST was repeated three times and the plate was shaken dry. Ten μl of anti-rabbit antibody conjugated with alkaline phosphatase (Miles Laboratories Ltd, Slough, Berks) was added to 5 ml of PBST containing 0.02% NaN_3 ; 50 μl of this mixture was dispensed into each well.

The plate was covered with aluminium foil and incubated for 1 h at 37°C. Again, wells were washed three times with PBST, shaken dry, and 50 μl of alkaline phosphatase substrate solution, containing p-nitrophenylphosphate (Sigma Chemical Co., St Louis, Missouri, USA) 1 mg/ml of 0.05M carbonate buffer, pH 9.8 with 0.001M MgCl_2 , was added to each well. The plate was then left uncovered at room temperature.

Interpretation of results. Plates were read by eye within 45 min. of the addition of substrate; a colour change from colourless to yellow was read as a positive result. Control wells containing cells + substrate, and substrate alone were always included in the test.

RESULTS

The following observations emerged from this study. (1) There was complete agreement between the results obtained in agar precipitation tests and in the ELISA for the strains tested. (2) The relative specificity of each of the grouping sera in the ELISA was assessed against known prototype strains and only when complete specificity was obtained with these prototypes were the unknown strains tested. It was necessary to do 5-9 absorptions on each of the sera before this degree of specificity was achieved. (3) The commercial antisera could be used at a maximum dilution of only 1 in 8 in the agar double-diffusion technique, whereas a dilution of 1 in 400 produced strong positive results in the ELISA. (4) An increase in incubation times for the separate stages would permit the use of even higher dilutions of serum in the ELISA procedure. However, plates should be read within the specified time to reduce the possibility of residual cross-reacting antibodies affecting the results.

DISCUSSION

Serological grouping of β -haemolytic streptococci in service laboratories is commonly performed by Lancefield's extraction of the carbohydrate antigen followed either by double

diffusion in agar gel or capillary precipitation tests. These methods are not only time consuming but, if tests are not performed with care, false-positive and false-negative results may occur. In recent times, grouping kits have become available commercially; although these are rapid and easily handled, they are without exception expensive, possibly precluding their use in some laboratories.

The ELISA procedure described in this paper is simple, sensitive and quick to perform. It has the same degree of reliability as gel-diffusion methods for grouping β -haemolytic streptococci, and is economical because antisera can be used in high dilutions. However, the fact that there is a certain degree of complexity in the initial standardisation of the assay may make it unattractive to laboratories dealing with only a few samples daily. The role of ELISA for the serological grouping of streptococci may therefore be limited to laboratories dealing with large numbers of specimens, and reference laboratories where quality control of sera may not be so difficult.

SUMMARY

A method of grouping β -haemolytic streptococci serologically by enzyme-linked immunosorbent assay is described. A comparison of this method with double diffusion in agar gel showed complete correlation of results when highly absorbed grouping sera were used.

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