The Laboratory Diagnosis of Whooping Cough by Fluorescent Antibody and by Culture Methods

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Attempts were made to hasten the identification of *Bordetella pertussis* by using the fluorescent antibody (FA) technique and to improve the isolation rate by culture methods. First, a comparison was made between the number of identifications of the organism by growth on Bordet-Gengou medium containing penicillin G and by staining smears of pharyngeal exudate with FA. Of 100 specimens, 29 were positive on culture and 46 were positive by the FA method. Five of the 46 were false positives. Second, 170 specimens were inoculated in quadruplicate on Bordet-Gengou medium containing either penicillin G or methicillin, and on Lacey medium containing either penicillin G or methicillin. In order there were 39, 41, 56 and 57 isolations. The isolation of *Bord. pertussis* on methicillin-containing media was easier than on penicillin-containing media. When culture and FA methods were compared, the most reliable was found to be the inoculation of specimens on Lacey medium.

On a essayé d'accélérer l'identification du *Bordetella pertussis* par la méthode des anticorps fluorescents (AF) et d'améliorer le pourcentage des isolements par la culture. On a d'abord procédé à une comparaison entre le nombre d'identifications du bacille obtenus par pousse du bacille sur le milieu de Bordet-Gengou contenant de la pénicilline G et celui utilisant la coloration de frottis d'exsudate pharyngiens avec la méthode des AF. Sur 100 spécimens, 29 se sont révélés positifs par culture et 46 ont été positifs par la méthode des AF. Cinq des 46 spécimens étaient faussement positifs. Ensuite, on a inoculé 170 échantillons, en quatre exemplaires, sur des millieux Bordet-Gengou contenant soit la pénicilline G, soit la méthicilline et sur des milieux de Lacey renfermant soit la pénicilline G, soit la méthicilline. Dans l'ordre, il y avait 39, 41, 56 et 57 isolements. L'isolement du *Bord. pertussis* sur les milieux contenant de la méthicilline a été plus facile que sur ceux contenant de la pénicilline. Après comparaison des méthodes par culture et par AF, la plus sûre s'est révélée être l'inoculation de spécimens sur le milieu de Lacey.

BORDETELLA PERTUSSIS requires from three to five days for laboratory identification, and failure to isolate it occurs frequently because of overgrowth of other organisms. It is particularly important in a children's hospital to be able to identify and isolate patients with whooping cough as quickly as possible in order to prevent cross-infection and to start appropriate therapy.

For these reasons it was decided to attempt to hasten the identification of *Bord. pertussis* both by introducing the fluorescent antibody (FA) technique and by trying to improve culture methods.

In the first phase of the project, specimens were examined from 100 patients suspected of having whooping cough and from 30 children without evidence of whooping cough (controls). Each specimen was inoculated on two Bordet-Gengou plates containing penicillin G, and smears were stained with fluorescent antibody prepared in the laboratory.

In the second phase, an attempt was made to diminish the growth of organisms other than *Bord. pertussis*. Specimens from 170 patients with suspected whooping cough were inoculated in quadruplicate on Bordet-Gengou medium containing either penicillin G or methicillin and on Lacey[1] medium containing either penicillin G or methicillin.

METHODS

Specimens

Specimens came from patients at The Hospital for Sick Children, Toronto, Ontario. They were taken from the nasopharynx of both infants and children by a method described by Auger.[2]

Production of Fluorescent Antibody

Antisera were produced in four 2-kg. white rabbits which were injected intravenously with a suspension of 10,000 million formol-killed *Bord. pertussis* organisms per ml. Doses were given at three-day intervals starting with 0.3 ml., then 0.6 ml., 0.9 ml., 2.0 ml., 2.4 ml. and finally 2.8 ml., making a total of 9.0 ml. over a period of 16 days. The rabbits were bled 10 days after the last injection. Conjugation of the antisera with fluorescein isothiocyanate was done according to the method described by Chadwick and Fothergill.[3] Brilliant staining of smears of *Bord. pertussis* was obtained with each antiserum diluted 1:16. Non-specific staining of other organisms frequently found in the nasopharynx was tested with all four conjugates. Six strains of *Staphylococcus aureus*, an often quoted offender in this respect, were stained only weakly by the selected antiserum. Pneumococci, diphtheroids and *Hemophilus influenzae* were not stained, but *Neisseria catarrhalis* and *Streptococcus pyogenes* stained with moderate brightness. Absorptions with bacterial powders failed to remove these reactions.

Fluorescent Antibody Staining and Examination of Smears

Direct staining of two smears and a blocking test of each specimen were done. For the direct stain, smears were fixed in acetone for 10 minutes and dried in air. Each preparation was covered with a drop of conjugated globulin. A damp container was used to prevent drying of the conjugate

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on the slides and the reaction was allowed to continue for 20 minutes at room temperature. The conjugate was then rinsed off with phosphate-buffered saline (pH 7.1) and the preparations were washed in three changes of the same solution with gentle agitation in a Coplin jar for a total period of 10 minutes. The preparations were placed gently between two layers of absorbent paper and then dried in air. The mountant used was glycerol-saline (90% glycerol, 10% phosphate-buffered saline). The principle of the blocking test is that staining with the conjugate should be inhibited by pre-treating the preparation with an unconjugated specific immune serum but not by pre-treating with a non-immune serum. Positive and negative control slides were included with every group of slides examined.

A Zeiss fluorescence miscroscope fitted with an HBO 200 lamp was used. The exciter filter used was BG 12 and the barrier filter cut radiation out below 510 millimicra. A cardioid condenser transmitted the radiation through buffered glycerol to the specimen which was viewed through 8 x oculars and a 100 x oil-immersion objective.

Culture Methods

For the first phase of the project, each specimen was inoculated on two Bordet-Gengou plates containing 0.185 unit penicillin G/ml. The plates were incubated at 35 to 36° C. for five days, with daily examination. Suspected colonies of *Bord. pertussis* were examined by Gram smear and by a slide agglutination test with commercial antiserum.

During the second phase of the project, each specimen was plated on Bordet-Gengou medium containing 0.185 unit penicillin G/ml., on Bordet-Gengou medium containing 4 μg. methicillin/ml., on Lacey medium containing 0.23 unit penicillin G/ml. and on Lacey medium containing 4 μg. methicillin/ml. The plates were incubated and examined as before. A level of 4 μg. methicillin/ml. was chosen because preliminary experiments showed that it caused an effective reduction in the growth of many penicillin G resistant organisms while still allowing free growth of *Bord. pertussis*.

IMPRESSION SMEARS

On a few occasions, impression smears were taken of microcolonies which had appeared after one to three days' incubation. The flat surface of a sterilized rubber stopper about 1 cm. in diameter was used. The stopper was gently lowered on the agar surface over the colony to be examined, care being taken not to break the surface of the medium. With the collected sample, three similar smears were made by pressing the stopper on glass slides. A direct stain and a blocking test were then done.

RESULTS

The results of the examinations of specimens from patients with suspected whooping cough both by fluorescent antibody and by culture techniques are given in Table I.

TABLE I.—SPECIMENS EXAMINED BOTH BY THE FLUORESCENT ANTIBODY METHOD AND BY CULTURE

Total tested	*Patient group*	*Control group*
Total tested	100	30
Number positive by FA	46	2
Number positive by culture on Bordet-Gengou	29	0
Number positive by culture, negative by FA	1	0
Number positive by FA, negative by culture	18	2

Of the 100 specimens from patients, 46 were judged positive by the FA technique and 29 were positive on culture. In only one instance was a positive culture obtained but *Bord. pertussis* not detected on the FA smears. Eighteen smears were positive by the FA method but negative on culture. On examination of the histories of these 18 patients, 11 had definite clinical evidence of whooping cough and so were probable true positives. Information was unavailable in two instances. Some of the remaining five may have been true positives and some false positives due to non-specific staining of other organisms. The percentage of false positives, then, could have been up to 10.8. Overall, however, 39 proved cases of whooping cough were detected by the FA technique, while only 29 were detected by culture, an increase of 34%. Of the 30 control specimens from children without evidence of whooping cough, two were positive by the FA method but none was positive on culture; thus the percentage of false positives was 6.6. Taking the test and control groups together, there were seven false positive results out of 130 (5.4%).

The examination of impression smears of microcolonies was not done routinely, but when it was done, the results indicated that it was a very useful additional technique. This was particularly true when the original FA smears were inconclusive.

The results of culturing, in quadruplicate, 170 specimens from patients with suspected whooping cough on Bordet-Gengou medium containing penicillin G, on Bordet-Gengou medium containing methicillin, on Lacey medium containing penicillin G and on Lacey medium containing methicillin are given in Table II.

TABLE II.—A COMPARISON OF POSITIVE CULTURES OBTAINED ON BORDET-GENGOU AND ON LACEY MEDIA CONTAINING EITHER PENICILLIN G OR METHICILLIN

Number of specimens tested in quadruplicate	170
Number of positive cultures on:	
Bordet-Gengou containing penicillin G	39
Bordet-Gengou containing methicillin	41
Lacey containing penicillin G	56
Lacey containing methicillin	57

All cultures isolated on Bordet-Gengou medium were also isolated on Lacey medium. It can be seen that there was an increase of 46% in isolations of *Bord. pertussis* on Lacey medium containing methicillin compared with isolations on Bordet-Gengou medium containing penicillin G, and the difference was significant (chi-square = 1.2, $p < .05$).

Although there was very little numerical difference in isolations on the same type of medium containing a different antibiotic, the absence of much of the penicillin G-resistant flora on methicillin-containing plates made isolation and identification easier and frequently faster. Fig. 1 shows two Bordet-Gengou plates inoculated with the same specimen. The medium on the left contained penicillin G and the medium on the right contained methicillin.

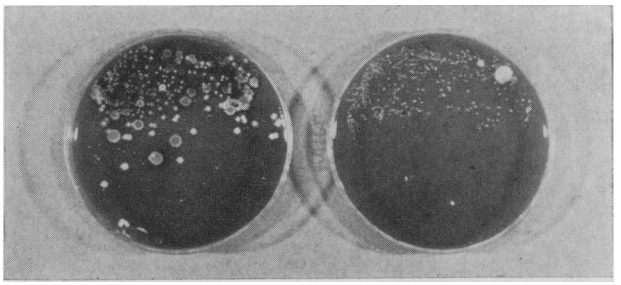

Fig. 1.—Isolation of *Bord. pertussis* on Bordet-Gengou medium containing penicillin G (left), compared with its isolation on the same medium containing methicillin.

Bord. pertussis organisms grew on both media but there was a heavy growth of other organisms on the penicillin G-containing medium, making isolation more difficult.

DISCUSSION

Compared with the conventional culture method on Bordet-Gengou medium, the FA method of diagnosing whooping cough has been found to be more sensitive (34% more "true positive" results) and certainly much faster. In about half of the positive specimens examined, organisms could be seen fluorescing which were readily identifiable as *Bord. pertussis* within an hour of receiving the specimen. The other positive preparations were not as easy to interpret, because organisms were often scarce and did not have a typical appearance. In these latter instances, impression smears of microcolonies were useful, especially before the organism could be identified by other means. Because the incidence of false positive results may have been as high as 10.8%, culture techniques were continued. The FA method is now used routinely as a basis for early preliminary reports.

In previous years, Bordet-Gengou medium containing penicillin G was the culture medium used in the laboratories of The Hospital for Sick Children for the isolation of *Bord. pertussis*. With the increased incidence of antibiotic-resistant organisms in the population, it was found that penicillin G was no longer exerting such an inhibitory effect. Colonies of *Bord. pertussis* were being overgrown by some commensal organisms as well as by many strains of *Staph. aureus*. Bordet-Gengou medium which contained methicillin was therefore introduced. At the same time, Lacey medium containing penicillin G as well as Lacey medium containing methicillin was examined.

Two facts came to light. The first was that the isolation rate was improved by 36.5 to 46% when Lacey medium was used. It should be pointed out that although a statistically significant difference could be proved only between Bordet-Gengou medium containing penicillin G and Lacey medium containing methicillin, a closer inspection of the figures involved shows that Lacey medium gave from 15 to 18 more isolations than Bordet-Gengou medium gave. The difference in the isolation rate depended on the type of medium used rather than on the antibiotic content. The second fact was that the advantage of methicillin over penicillin G was in the ease and speed of isolation due to the absence of methicillin-sensitive organisms.

Although the FA method was 34% more sensitive in the detection of *Bord. pertussis* than culture on Bordet-Gengou medium, it was still less sensitive than culture on Lacey medium. Perhaps if a reliable means of preventing false-positive results were available, the speed of diagnosis by the FA method would be an outweighing factor in its favour. To the present time, however, inoculation of specimens on Lacey medium has been found to be the most reliable laboratory method of diagnosing whooping cough.

One disadvantage of Lacey medium was encountered. Colonies of *Bord. pertussis* grown on Lacey medium sometimes were found not to emulsify well enough to do agglutination tests. In these instances it was necessary first to subculture on Bordet-Gengou medium or to use growth already present on a Bordet-Gengou plate.

A valuable inclusion in methicillin-containing Lacey medium would be a substance capable of inhibiting the growth of *Escherichia coli* and *Klebsiella pneumoniae* while at the same time neither preventing the growth of *Bord. pertussis* nor inhibiting the action of methicillin.

SUMMARY

Specimens of pharyngeal secretions from 100 patients with suspected whooping cough and from 30 children without evidence of whooping cough were examined both by the FA method and by culture on Bordet-Gengou medium with penicillin G. The number of patients' specimens found positive by the FA technique was 46. Of these, five were false positive, two were inconclusive, and 39 were true positive results, By culture, only 29 positive results were obtained. Two control specimens were found positive by the FA technique, both of which were probably false. By culture, no positive results were obtained. The FA technique

was more sensitive and much faster than the culture method but, because of false positive results that could not be eliminated, it was found to be most useful in giving early preliminary results.

Specimens from 170 patients with suspected whooping cough were inoculated in quadruplicate on Bordet-Gengou medium containing either penicillin G or methicillin, and on Lacey medium containing either penicillin G or methicillin. There was an increase in isolations of *Bord. pertussis* on Lacey medium compared with Bordet-Gengou medium. In addition, isolation was greatly facilitated by the use of methicillin.

When culture and FA methods were compared, the most reliable was found to be the inoculation of specimens on Lacey medium containing methicillin.

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INTERNATIONAL MEDICINE

Wings of Mercy: Medical Aid to Honduras

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THE project of medical aid to the Republic of Honduras described in this communication was originated, sponsored and managed by the River Oaks Baptist Church, Houston, Texas, and its ministers, the Rev. W. S. Failing and the Rev. Guy Bevil, Jr. Support was provided by several organizations including AMDOC Inc.,* the Flying Physicians' Association and the Evangelical Medical Missionaries Aid Society of Canada (EMMAS) as well as church groups in the United States.

The purpose of the trip was to carry out an intensive course of inoculations (BCG and smallpox) across a strip of Honduras from the Caribbean Sea to the Pacific Ocean during a period of three weeks in the summer of 1965, to demonstrate new techniques to medical and other health personnel in that country, and to carry a Christian witness to all concerned. Dr. G. R. Bourne of Toronto and I are members of the Flying Physicians' Association and of the Evangelical Medical Missionaries Aid Society. The opportunity to take part in this massive international health activity (which was given the title *Amigos de Honduras*), to show the flag for Canadian physicians, to demonstrate Christian concern for the troubles of others, and to undertake an interesting flight, were all factors in our participation.

We travelled from Toronto to San Pedro Sula, Honduras, and returned in Dr. Bourne's twin-engined Beech Bonanza Aircraft. Dr. Bourne was the pilot and was accompanied by his wife and two children, Mrs. Flatt and myself.

On July 1, 1965, we took off from the Toronto Island Airport at about 7:05 a.m., flew direct to Cleveland, landed there at 8:15 a.m. and cleared U.S. customs. Subsequent stops were made at Memphis, Tennessee, and Houston, Texas. At Houston we telephoned the Rev. W. S. Failing, the chairman of the sponsoring committee, who met us at the airport and gave us much worth-while information about the project. The weather was sunny and very warm. Later the same day we took off for Brownsville, Texas, which is just north of the Mexican border and the Rio Grande. We stayed there that night. Brownsville is a pleasant small American city with much Spanish and Mexican architecture. Mr. Guy Bevil, Sr., who is the Honduran consul there, stamped our passports for admission into Honduras.

Our next overnight stops were at Vera Cruz and Carmen, Mexico. The city square in Vera Cruz is very beautiful and ornate. The open streetcars were quaint and the people were very friendly. Carmen is a beautiful small city on an island situated at the southeastern end of the Gulf of Mexico. Carmen is only about 275 miles from Vera Cruz, but we had to stop there because of severe thunderstorms. The following day we took off and after stops at Chetumal, Mexico, and Belsize, British Honduras, we reached San Pedro Sula, which is the second largest city in Honduras. San Pedro Sula is situated in the north-west sector of the Republic about 30 miles from the coast. After clearing customs and immigration we were taken to our hotel. The weather was very warm and balmy.

The Republic of Honduras is a military dictatorship headed by Brigadier-General Oswaldo Lopez Alrellano, who appears to be benevolent and is

*AMDOC Inc. (American Doctor) was founded in 1964 by Paul Williamson, M.D.

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