Serotyping and Detection of *Haemophilus pleuropneumoniae* by Indirect Fluorescent Antibody Technique

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ABSTRACT

This report provides a description and evaluation of the indirect fluorescent-antibody technique for serotyping and detecting *Haemophilus pleuropneumoniae*.

The indirect fluorescent-antibody technique was serotype-specific when reference strains and sera were tested. Sixty-five field isolates were serotyped by indirect fluorescent-antibody technique and belonged to types 1, 2 and 5. Two isolates did not fit any of the five established types. Twenty-three of the 65 isolates were also typed by immunodiffusion and the two tests agreed completely. *Haemophilus pleuropneumoniae* was detected in freshly-fixed impression smears from 12 lungs having acute pleuropneumonia. The identity of the bacteria was subsequently confirmed by cultural procedures. Fixed smears of cultured *Haemophilus* bacteria can be stored or mailed at -20° to +24°C without losing stainability.

RÉSUMÉ

Cet article présente une description et une évaluation de la technique d'immunofluorescence indirecte, comme moyen de détecter *Haemophilus pleuropneumoniae* et d'en identifier les sérotypes. Cette technique s'avéra spécifique, lors de la vérification des souches et des antisérums de référence. On l'utilisa ensuite pour déterminer le sérotype de 65 souches isolées de cas spontanés de la maladie; elles appartenaient aux sérotypes #1, #2 et #5, sauf deux qui ne correspondaient à aucun des cinq sérotypes actuellement connus. La détermination du sérotype de ces 63 souches, par l'immunodiffusion en gélose, donna les mêmes résultats que l'immunofluorescence indirecte. On dé-cela *H. pleuropneumoniae* dans les impressions préparées à même 12 poumons qui présentaient des lésions de pleuro-pneumonie aiguë. L'examen bactériologique permit de confirmer l'identité de cette bactérie. La fixation d'impressions préparées à partir de colonies de *H. pleuropneumoniae*, permet de les entreposer ou de les envoyer par la poste, à des températures variant de -20° à +24°C, sans qu'elles perdent leur capacité de réagir à la coloration.

INTRODUCTION

Pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae* is world-wide in distribution and results in severe losses to the swine industry (8, 9). Five serotypes, 1-5, of *H. pleuropneumoniae* are recognized (1). A few additional isolates from porcine pleuropneumonia are serologically, biochemically and genetically distinct from serotypes 1-5 and may constitute a separate species (5). Serotypes 1, 3, 4 and 5 seem to be predominant in North America, whereas serotype 2 is the most significant in Europe (4). Serotyping is based on whole-cell tube agglutination and immunodiffusion tests (1, 2). The serotype-specific antigens are heat-stable and considered to be cell-wall associated (3).

Serotyping of field isolates is important when considering immunoprophylaxis and serological diagnosis as tools in the control of the disease. Both agglutination and precipitation tests require that antigens are prepared after the bacterium has been isolated and cloned.

The purpose of this study was to evaluate the effectiveness of an indirect fluorescent-antibody technique (IFAT) for the detection and serotyping of *Haemophilus pleuropneumoniae* as this test was expected to be far more rapid than both the agglutination and precipitation tests.

MATERIALS AND METHODS

BACTERIAL STRAINS

The reference strains for the 5 serotypes listed in Table I were used. In addition, 65 isolates of *H. pleuropneumoniae* from pigs with

TABLE I. Reference Strains of Five Serotypes of *H. pleuropneumoniae* and Homologous Titres of Antiserum

Strain[a]	Serotype	Homologous titre in IFAT
Shope 4074	1	160
S 1536	2	640
S 1421	3	160
M 62	4	160
K 17	5	160

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pleuropneumonia were serotyped. All strains and isolates were cultured on calf blood agar containing 0.1% NAD[1] and stored at -70°C in trypticase soy broth containing 0.1% NAD and 0.25% Noble agar.

LUNG TISSUE

A total of 12 specimens of lung tissue was submitted, frozen. The lungs had typical changes of acute pleuropneumonia. All tissues were cultured and impression smears prepared for IFAT.

ANTISERA

Antisera were produced in rabbits against the five reference strains. One Roux flask containing 150 mL trypticase soy agar and 0.1% NAD was inoculated with a suspension of bacteria from a plate culture. After six hours incubation at 37°C, 100 mL of trypticase soy broth with 0.1% NAD was added and the culture checked for purity. After another 12 hours, the bacteria were harvested by centrifugation, washed three times in saline and concentrated by freeze-drying in preweighed vials. The antigen was reconstituted with saline containing thimerosal (1:10,000)[2] to obtain a concentration of 5 mg antigen per mL. Three white New Zealand rabbits were inoculated twice weekly with each antigen. Initially, 0.5 mL was given subcutaneously. The subsequent doses were given intravenously and increased with 0.5 mL up to 3.0 mL. The rabbits were exsanguinated when their sera contained antibodies reacting against homologous antigen in the immunodiffusion test.

INDIRECT FLUORESCENT ANTIBODY TECHNIQUE

Thin smears were made on microscope slides from cultures or lung tissue. The smears were air-dried and fixed in 10% buffered formalin for ten minutes. The slides were rinsed under running tap water and air-dried. One drop of serum dilution was applied to each smear and incubated in a moist chamber at 35°C for 45 minutes. After excess serum was drained off, the slides were washed in phosphate buffered saline (PBS) pH 7.5 for ten minutes followed by a brief wash under tap water. One drop of goat-anti-rabbit serum conjugated with fluorescein-isothiocyanate[3] and diluted 1:20 was applied to each smear and incubated for 30 minutes at 35°C. The slides were washed as before and mounted in glycerol-PBS (9+1). Fluorescence was examined at 1000 times magnification with a Zeiss Universal microscope equipped with a HBO Mercury lamp, the epi-fluorescence condenser III RS, the BG-38 filter and the KP490 interference filter for excitation, the FT580 chromatic beam splitter mirror and the LP520 barrier filter. The optimal conjugate dilution was determined from a checkerboard titration using strain Shope 4047 (serotype 1) as antigen and serial twofold dilutions of homologous serum and conjugate beginning at 1:10. The dilution giving the strongest specific staining of bacteria and the weakest background of fluorescence was chosen.

TITRATION OF REFERENCE SERA

Pre- and postimmunization sera were titrated against homologous strains, using serial twofold dilutions from 1:20-1:1280. The reference sera were also tested in 1:20 dilutions against all heterologous reference strains. Fluorescence was rated on a scale of 0, +, ++ and +++ with only ++ and +++ recorded as positive reactions characterized by brilliant peripheral fluorescence. A weakly fluorescing outline of bacteria was marked +.

SEROTYPING OF ISOLATES

Three slides of each isolate were tested with serotype 1 and 5 antisera and normal rabbit serum diluted 1:20 respectively. If all were negative, additional slides were tested with serotype 2, 3 and 4 antisera.

DETECTION OF *H. PLEUROPNEUMONIAE*

Three smears of each lung were stained with 1:20 dilutions of serotype 1 and 5 antisera and normal rabbit serum respectively. The lungs were cultured on blood agar with NAD and the isolates were purified and typed by IFAT as described above.

EFFECT OF STORAGE

Bacterial smears and lung smears were fixed and stored at room temperature for one week and then stained. For comparison, freshly prepared smears were fixed and stained.

IMMUNODIFFUSION

Bacteria were cultured and harvested similar to immunization antigens. Distilled water and 90% phenol were added in amounts equal to 20 times the wet weight of sedimented bacteria. The suspension was heated to 65°C for 20 minutes and continuously stirred. After cooling, the preparation was centrifuged at 4,000 g for 20 minutes and the water phase harvested. This procedure was repeated and the two water phases combined and dialysed for 48 hours at 4°C against distilled water. The extracts were concentrated five to six times by use of polyvinylpyrrolidone.[4]

Immunodiffusion was carried out in gels consisting of 1% agarose in veronal buffer (pH 8.6). Seven wells, one central and six peripheral, of 2 mm in diameter, were punched in the gel by use of a template.

Antigen extract of each strain to be tested was put in the central well and antisera were added to the peripheral wells. The gels were incubated in a moist chamber and read after two and three days.

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[1]Nicotinamide adenine dinculeotide, Eastman Kodak Co., Rochester, N.Y.
[2]Nutritional Biochemicals Corporation, Cleveland, Ohio.
[3]Miles Laboratory, Elkhart, Indiana.
[4]Matheson, Coleman and Bell, Norwood, Ohio.

RESULTS

TITRATION OF REFERENCE SERA

The homologous titres of the reference sera are shown in Table I. No cross-reactions between serotypes were found in serum diluted 1:20. Figure 1 shows a +++ fluorescence obtained in a homologous reaction.

All reference sera had precipitating antibodies against homologous, but not against heterologous antigens.

SEROTYPING OF ISOLATES

The IFAT identified 63 *Haemophilus* strains out of 65 isolated from lungs of pigs with pleuropneumonia (Table II). Of these strains, 23 were tested by immunodiffusion and 21 serotyped successfully. There was complete agreement between the immunodiffusion results and the results of IFAT.

DETECTION OF *H. PLEUROPNAUMONIAE*

Smears of lungs with pleuropneumonia generally had between five and 20 bacteria per microscope field at 1000 times magnification (Fig. 2). Of 12 lungs, 11 had serotype 1 and one had serotype 5. These results were confirmed in all cases when the lungs were cultured and the isolates serotyped.

TABLE II. Results of Serotyping *H. pleuropneumoniae* Isolates by IFAT and Immunodiffusion

	Strains typed by IFAT alone	Strains typed by IFAT and Immunodiffusion	Total
Serotype 1	34	11	45
Serotype 2	—	1	1
Serotype 5	8	9	17
Not typeable	—	2	2
Total number tested	42	23	65

EFFECT OF STORAGE

There was no difference in fluorescent intensity or specificity between freshly-fixed smears and smears which had been fixed and then left at room temperature for a week. The lung smears, on the other hand, gave less distinct and more smudgy fluorescence after a week of storage.

DISCUSSION

Supernatants from centrifuged suspensions of *H. pleuropneumoniae* contain serotype-specific antigens as well as antigens shared between serotypes (2). Phenol-water-extracted antigens are serotype-specific when tested by immunodiffusion (3). Serotyping of *H. pleuropneumoniae* is important for several reasons: 1) it aids in epidemiological studies on transmission and distribution of infection and disease; 2) effective immunoprophylaxis may only be possible with vaccines prepared from homologous serotype strains. Although experimentally-infected pigs seem to develop immunity against heterologous challenge strains (7), cross-protection is not satisfactory with formalin-inactivated adjuvanted vaccine preparations; 3) serotyping is important in serodiagnostic studies as antibodies are best detected with homologous antigen (6).

Haemophilus pleuropneumoniae can undergo S-R dissociation. Rough strains cannot be typed by agglutination reactions (2). Typing by immunodiffusion is cumbersome and time-consuming. Our study showed that IFAT is serotype-specific and therefore can replace agglutination or immunodiffusion tests. The advantages of IFAT are: 1) it is rapid; results can be obtained after three to four hours; 2) culture is not necessary as bacteria in fresh impression smears will stain; 3) bacteria fixed onto slides can be mailed to the laboratory and typed. This is based on our findings that fixed smears could be stored for a week at room temperature without losing stainability. More experience is needed to ascertain the reliability of stored fixed impression smears of lung tissue.

The IFAT may be useful for the mere detection of *H. pleuropneumoniae* infections. However, studies on possible cross-reactions with other bacteria commonly associated with pneumonia in pigs

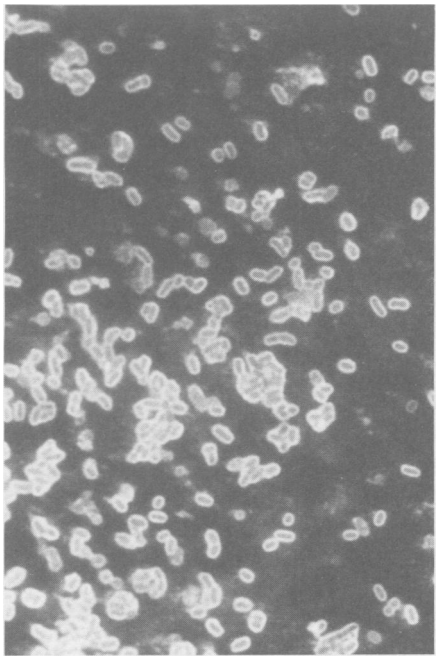

Fig. 1. Brilliant fluorescence of culture smear of *H. pleuropneumoniae*, strain Shope 4074 stained with indirect fluorescent antibody technique.

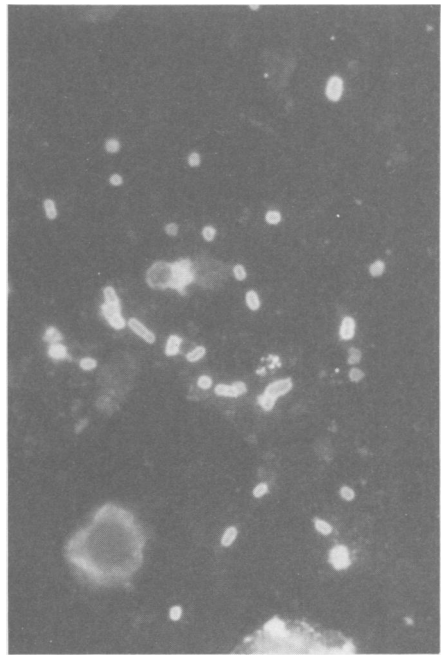

Fig. 2. Bacteria in lung smear stained with indirect fluorescent antibody technique using serotype 1 antiserum.

(e.g. *Pasteurella multocida, Bordetella bronchiseptica,* streptococci, etc.) are needed in order to evaluate the reliability of IFAT.

The IFAT seems to stain surface-associated antigens (Fig. 1). The same antigens may very well be extracted by the phenol-water procedure, which would explain the agreement between IFAT and immunodiffusion tests.

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