FACTORS AFFECTING THE INDIRECT IMMUNOPEROXIDASE STAINING OF INFECTIOUS BURSAL DISEASE VIRUS ANTIGENS IN CELLS

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SUMMARY

THE application of the Immunoperoxidase (IP) antibody technique for the definitive diagnosis of Infectious Bursal Disease (IBD) in chickens has earlier been described (Okeke and Lang, 1982). During that study it was noted that the indirect method of IP was more sensitive in the demonstration of IBD viral antigens than the direct method. Consequently, more information was sought on the specific technical parameters and the consequences that resulted by deviations from the codified procedures employed in the Indirect technique with the aim of obtaining an even better result.

Studies of pH, serum dilutions, serum incubation time, conjugate dilutions, conjugate incubation time and developing time were conducted to find out what influence such factors could have on the final staining product.

INTRODUCTION

Since Coons & Kaplan, (1950) introduced the immunofluorescence technique (IFT) for the demonstration of cellular particles or antigens, a great deal of progress has been made in immunocytological methods. Fluorescent dyes (Coons, 1959), radioactive substances (Berenbaum, 1959) and heavy metals (Pepe, 1961) have been used as markers.

The use of enzymes as markers was later introduced by Nakane and Pierce (1967), Avrameas and Uriel (1966). In these techniques the enzyme was first covalently linked to the antibody, then the enzyme-antibody conjugate was allowed to react with the cellular antigen. The sites of antigen — conjugate reactions were then revealed by cytochemical methods appropriate to the conjugated enzyme.

The standard indirect staining method employed in the demonstration of these antigenic sites was that of Nakane and Kawaoi (1974). In this technique the sections were placed in an airtight box, covered with a specific antiserum and incubated at 37°C for 1 hour. The antiserum was removed by rinsing in at least eight changes of PBS was drained and the sections covered with labelled rabbit anti-chicken IgG and incubated again in a humidified box at 37°C for 1 hour. The conjugate was then rinsed in 3 changes of PBS. The PBS was drained and the slide placed in a developing solution for 10 minutes. It was then rinsed in phosphate sucrose buffer, dehydrated in ethanol, cleared in xylene and mounted in DPX mountant.

For the staining of the conjugate in a developing solution the standard staining method involved the use of phosphate-sucrosebuffer (PSB) pH 7.4 prepared by mixing 20 ml 0.1 MKH_2PO_4 with 80ml O.1$Mna_2HPO.\ 7H_2O$ and adding to it 3.42g sucrose (Miller, Karnovsky & Diamandopoulos 1974).

To 100ml PSB pH 7.4 was added 50mg diaminobenzidine (DAB) tetrahydrochloride (sigma chemical). This was kept in the dark and shaken intermitently over a

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30 — 45 minute period and then filtered to remove any undissolved DAB. To the filtrate was added just before use O.03ml of a 30% solution of Hydrogen peroxide (H_2O_2) to give a final concentration of 0.009% of H_2O_2, kept in the dark because it darkens on standing, particularly when exposed to light. The conjugate was allowed to develop in this solution for 10 — 20 minutes.

During studies on the IP technique for diagnosis of IBD (Okeke and Lang, 1982), it was observed that the standard staining procedure of Nakane and Kawaoi (1974) did not always produce desirable results. It became necessary therefore to study the various aspects of the staining process in order to determine the optimum conditions for IP staining. In this report, we present the results of studies on the effect of pH, various concentration of reagents, variations in length of incubation and development on indirect IP staining of IBD virus infected cells.

MATERIALS

Virus strain and antiserum: was Agent 2571 and its anti-serum defined earlier (Okeke and Lang, 1982).

RESULTS

The following results were obtained as a result of deviations from the codified procedures:-

(a) Effect of pH variations

Citrate — phosphate buffer of pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 and phosphate saline (PBS) of pH 7.0, 7.5 and 8.0 were prepared. The infected cell culture monolayers were stained by the standard method for immunoperoxidase staining except that DAB solutions and all washings were done using buffer (PBS or citrate) of the pH under test. The stained cultures were examined under the microscope and the effect of each pH level on the staining quality was scored for intensity, clarity and contrast. The pH level at which only the virus infected cells were stained intensely enough to be observed with ease under the light microscope was regarded as the optimum pH for staining.

The effect of pH on staining quality is shown in Table 1. At pH 4.5 and lower the monolayer appeared cloudy and the staining was not specific. AT pH 5.0 to 5.5 stained infected cells could hardly be differentiated from the uninfected cells; the monolayer was hazy and specificity was fair.

TABLE *1

The Effect of pH Variations on the Immunoperoxidase Staining of Agent 2571 Infected Chicken Embryo Liver (CEL) Monolayer

Buffer	*pH*	*Intensity of Staining of Infected Cells*	*Clarity of Cell Outline*	*Contrast between Specific and Background Staining*
CIT-PO_4	4.0	0	Cloudy	None
	4.5	0	Cloudy	None
	5.0	1+	Hazy	Fair
	5.5	1+	Hazy	Fair
	6.0	2+	Clear	Moderate
	6.5	3+	Very clear	Good
PBS	7.0	3+	Very clear	Good
	7.5	3+	Very clear	Good
	8.0	3+	Very clear	Moderate

Legend:

Intensity 3+ = very dark staining
Intensity 2+ = moderate staining
Intensity 1+ = fairly dark staining
Intensity 0 = no staining.

At pH 6.0 the cell outlines could be clearly discerned, the intensity and specificity were moderately good. From pH 6.5 to 7.5 the cell outline could be seen very clearly and the intensity and contrast between specific and background staining was good. At pH 8.0 the cell outlines were still well defined but uninfected controls stained non-specifically. These observations indicate that for optimum results the correct staining pH of solutions should not be less than 6.5 but not exceed 7.5.

(b) Effect of Serum dilutions

Serial fourfold dilutions of the anti-Agent 2571 serum were prepared from 1:4 to 1:16384 in PBS, pH 7.4. Each serum dilution was employed separately in the indirect staining of infected Agent 2751 — monolayers, all other reagents were the same. The highest dilution of serum that gave a specific staining reaction of acceptable intensity and minimum background staining was selected as the optimum dilution in which the serum must be used for best results.

Table 2 shows the effect of antiserum dilutions on indirect IPT staining of cell monolayers infected with Agent 2571. The serum in its undiluted form did not stain as intensely as the 1:2 dilution. The staining intensity was best between 1:2 and 1:64 dilutions of immune serum, but decreased progressively thereafter such that at 1:2048 only the unstained, uninfected

TABLE 2:

The Effect of Dilution of Anti-2571-Serum on the Immunoperoxidase Staining of Agent 2571 Infected Chicken Embryo Liver (CEL) Monolayer

Cell Monolayer	Serum	Conjugate	Reciprocal of Serum Dilutions and Contrast Obtained between Specific and Background Staining													
			1	2	4	8	16	32	64	128	256	1024	2048	4096	8192	16384
UNINFECTED	ANTI-2571	RAC-IgG-HRPO	0								ND					
INFECTED	ANTI-2571	RAC-IgG-HRPO	2+	3+	3+	3+	3+	3+	3+	2+	2+	1+	1+	1+	0	0
INFECTED	NEG. SERUM	RAC-IgG-HRPO	0								ND					
INFECTED	ADSORBED S.	RAC-IgG-HRPO	0								ND					
INFECTED	ANTI-CRAWLEY	RAC-IgG-HRPO	0								ND					
INFECTED	ANTI-2571	UNCONJUGATED RAC-IgG.	0													

cells could be seen whereas infected cells could no longer be seen in cytopathic areas.

(c) *Effect of Serum incubation time*

The incubation time after addition of antiserum to the coverslip was studied for periods of 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes at 37°C in humid chamber. The results are shown below in tabulated form:

(d) *Effect of Conjugate Dilutions*

Serial two-fold dilutions of conjugate were prepared from 1:2 to 1:256 in PBS pH 7.4. Infected cell culture monolayers were stained with the optimum serum dilution (1:64) (Table 2) and then with the conjugate dilution under test, all other reagents remaining constant. The optimum dilution of the conjugate considered best for routin staining procedures was the highest dilutio that gave a specific staining reaction with undiminished intensity and with minimum background staining.

Table 3 shows the effect of conjugate dilutions on the indirect IPT of cell monolayers infected with "Agent 2571." The conjugate could

TABLE 3:

The Effect of Dilution of Rac-IgG-HRPO Conjugate on the Staining of Agent 2571 Infected Chicken Embryo Liver (CEL) Monolayer

Cell	1:64 Serum	Conjugate	Reciprocal of Conjugate Dilutions and Intensity of Staining								
			0	2	4	8	16	32	64	128	256
UNINFECTED	ANTI-2571	RAC-IgG-HRPO	0					ND			
INFECTED	ANTI-2571	RAC-IgG-HRPO	3+	3+	3+	3+	3+	2+	1+	0	0
INFECTED	NEG. CONTROLS	RAC-IgG-HRPO	0					ND			
INFECTED	ANTI-2571	UNCONJUGATED RAC-IgG	0					ND			
INFECTED	ANTI-2571	ADSORBED CONJUGATE	0					ND			

stain up to a dilution of 1:64 (1+) after which the staining ability was lost. The intensity of background staining faded gradually with the conjugate dilution, but the full staining intensity of infected cells remained the same (3+) up to a dilution of 1:16, after which it was slightly reduced (2+). Bursal sections and infected cell culture monolayers were satisfactorily stained at this 1:16 dilution of the conjugate.

(e) ***Effect of Conjugate incubation time: Infected Coverslips were first incubated with specific anti-serum***

After washing off residual serum thoroughly in several changes of PBS, pH 7.4, coverslips were covered with the conjugate at 1:16 dilution and incubated for 4,10,15,20,25,30,45,60,90 and 120 minutes at 37°C in a humidified chamber. The following staining results were obtained:

TABLE 4:
Serum Staining Time:

Incubation	*Time*	*Observation: Specificity of Staining*
5 — 15	minutes	Poor
20 — 25	minutes	Fair
30 — 60	minutes	Good or Excellent. Little background Staining
90	minutes	Poor. Increased background staining
120	minutes	Very Poor. Extensive non-specific staining of uninfected cells.

TABLE 5:
Conjugate Staining Time

Incuba tion	*Time*	*Observation: Specificity of Staining*
5—15	minutes	Poor. Infected cells cannot be differentiated from uninfected cells
20—25	minutes	Fair. Infected cells begin to stain faintly
30—60	minutes	Good. Little background staining
90—120	minutes	Poor. Too much background staining.

(f) Effect of Hydrogen peroxide (H_2O_2) concentration

Hydrogen peroxide was purchased as a 3% (W/V) solution in water. Dilutions were prepared from the stock solution to produce 0.3, 0.15, O.O5, O.02, 0.1, 0.005 and 0.0005 per cent H_2O_2. The effects of the different concentrations of H_2O_2 on the staining intensity and specificity of IP — staining were studied.

The results showed that increasing concentration of H_2O_2 progressively increased the intensity but decreased the specificity of staining. The 0.01% concentration of H_2O_2 was regarded as optimum for the staining procedure.

(g) Effect of Developing time

Virus infected tissue were incubated in the presence of DAB containing 0.01% H_2O_2 for 5, 10, 15, 20, 25, 30 and 60 minutes and the staining intensity and specificity was studied for each preparation. The incubation was done at room temperature and in the dark to prevent the effect of light on DAB. The results indicated that sections could be incubated in the developing solution for up to 20 minutes without any significant change in the staining specificity. At 5 minutes normal controls began to show some darkening which became most intense after 60 minutes. The optimum staining intensity was attained at 10 minutes incubation.

DISCUSSIONS

The routine application of a diagnostic test requires also that it can be reliably carried out by laboratory technicians. The IP-test does not demand very critical experimental parameters, except the necessity of a good, monovalent specific antiserum. The functionally acceptable pH range of 6.5 to 7.5 is wide. Hanon, *et al* (1975), Eckert & Simpson (1975) prefer to work in the acid (pH 6.8), while Miller, *et al* (1974) recommend alkaline (pH 7.5) conditions. The present study indicates that pH is notcritical, and acceptable specificity can be obtained between pH 6.5 and 7.5. The dilution of the anti-viral serum will not affect the diagnostic interpretation of the staining if carried out moderately beyond the optimum range; only the staining intensity will be reduced. The use of antiserum of dilutions up to 1:64 and higher increased the quality of staining by reducing the background staining considerably so that infected cells alwlays stained much darker than non-infected cells. As with other serological procedures, the use of undiluted serum affects the test results by an excess of non-immunological proteins, which tend to overshadow the specific immune complexes. Similarly the serum incubation time of 45 minutes is very practical for daily routine and still leaves enough flexibility (± 15 minutes) without annoying consequences. This study indicates that serum incubation time can be shortened to 30 minutes with excellent result.

The optimum conjugate dilution has to be determined for each batch, but the diluted conjugate was stable for at least six months when stored at -20°C and at 4°C for a day, but no attempts were made to use the diluted conjugate on subsequent days. Refreezing of the diluted conjugate is not recommended and was found to be detrimental to the test in this study. The conjugate was best used at a dilution of 1:16 for 45 minutes at 37°C.

The concentration of H_2O_2 in the developing reagent was routinely 0.01%, a value close to the upper limit of the range established in this study (0.0005% to 0.02%) and within the range of 0.005 to 0.05% of Weir, Pretlow, Pitts and

Williams (1974), of 0.006 to 0.02% given by Nakane and Yasuda (1975) and 0.075 to 0.188% for the enzyme assay developed by Lyr (1975). Excess of H_2O_2 is said to cause reaction with tissue hemeenzymes other than peroxidase (Berenbaum, 1959).

The developing time of the staining of 10—30 minutes is a wider value than the recommendations of Nakane and Kawaoi, (1974) of 10 — 20 minutes. Weir *et al,* Pretlow, Pitts and Williams (1974) preferred to incubate sections first in DAB alone for 30 minutes then for 2 minutes in DAB with H_2O_2. This modification was not found better than the original method of Graham and Karnovsky, (1966).

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