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Characterization of a Screening Test for Diphtherial Toxin Antigen Produced by Individual Plaques of Corynebacteriophages

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A passive immune hemolysis assay has been developed to detect diphtherial toxin produced in individual plaques of tox^+ corynebacteriophages. This assay permits rapid screening of large numbers of corynebacteriophages for their ability to code for diphtherial toxin or related antigens. The specificity of the assay and its potential usefulness for genetic studies of toxinogenesis have been demonstrated with well-characterized tox^+ and tox^- laboratory strains of corynebacteriophages.

The structural gene for diphtherial toxin is present in phage β^{tox^+} and in other tox^+ corynebacteriophages, and toxinogenesis in *Corynebacterium diphtheriae* provides one of the best-studied examples of phage conversion (1, 27). The biochemistry, genetics, physiology, and immunology of diphtherial toxin have been extensively investigated and have provided important models for research with other microbial toxins (3, 23, 24).

Many in vitro assays for diphtherial toxin have been developed, including immunological tests (5, 8, 9, 12, 22), tissue culture cytotoxicity tests (6, 20, 28), and enzymatic assays (4, 7, 13). The available assays vary greatly in sensitivity, ease of performance, and cost, but several of these methods have been used successfully for phenotypic characterization of wild-type, mutant, and recombinant strains of corynebacteriophages in previous studies (10, 11, 16, 18, 20, 21, 26, 29, 30). In all of these studies, however, it was necessary at some point to obtain cloned isolates of corynebacteriophages and to perform individual analyses with them. Thus, the time and effort required to isolate large numbers of samples either from single plaques or from single colonies of lysogenic bacteria imposed practical limitations on the total numbers of phage clones that could be tested.

In the present study we report the development and characterization of a visually scorable assay based on the principle of reversed passive immune hemolysis. It has been used successfully to detect diphtherial toxin antigen in individual plaques of tox^+ corynebacteriophages developing in lawns of *C. diphtheriae* in agar medium. In this way, the need to isolate clones of phages from individual plaques before they can be screened for toxinogenicity has been eliminated. The potential usefulness of this assay for the isolation of *tox* mutants or *tox* recombinants in future genetic studies of corynebacteriophages was illustrated by control experiments with well-characterized laboratory strains of corynebacteriophages and corynebacteria.

MATERIALS AND METHODS

Bacteria and bacteriophages. *C. diphtheriae* C7s(−)$^{tox^-}$, hereafter designated C7, is nontoxinogenic, is sensitive to phage β^{tox^+}, and is the routine indicator strain and propagating host for the corynebacteriophages used in this study (10, 11). *C. diphtheriae* C7/β^{vir}/β^{hc} is resistant to phage β and serves as the selective indicator for mutant or recombinant phages with extended host range of the *hh'* type (10, 11). The strains of phages β^{tox^+}, $\beta^{tox\text{-}1}$, $\beta^{tox\text{-}30}$, $\beta^{tox\text{-}45}$, $\beta^{h_1 tox^- h'_2}$, $\beta^{h_1 tox^+ h'_2}$, and γ^{tox^-} maintained in our laboratory have been described previously (10, 11). Phage $\beta^{tox\text{-}197}$ (originally called β^{197}) was provided in the lysogenic strain *C. diphtheriae* C7($\beta^{tox\text{-}197}$) by T. Uchida (30). Phage $\beta^{tox^+}_{ct1}$ has been reported to be partially insensitive to the inhibitory effect of iron on toxin production (21) and was provided by J. R. Murphy in the lysogenic strain *C. diphtheriae* C7($\beta^{tox^+}_{ct1}$). Procedures for routine cultivation of bacterial strains and for propagation of phage stocks were as described previously (10, 11).

Diphtherial toxin, toxoid, and antitoxin. Partially purified diphtherial toxin was purchased from Connaught Laboratories (Toronto, Canada) and purified to homogeneity (12). A sample of purified toxin was converted to toxoid by published methods (25). Diphtherial antitoxin (150 antitoxin units per ml) was obtained after hyperimmunization of a goat by repeated intramuscular injections of 1-mg samples of diphtherial toxoid in complete Freund adjuvant. Immunoglobulin G (IgG) was purified from the hyperimmune goat antitoxin by ammonium sulfate precipitation followed by diethylaminoethyl-cellulose chromatography by using published methods (17). The

purified IgG fraction (25 antitoxin units per mg) was dialyzed against normal saline and stored at −80°C. Antitoxin titers were determined by intracutaneous neutralization tests in rabbits and were based on comparison of the test samples with a reference equine antitoxin (lot DP 1252; 11.25 antitoxin units per mg; Wellcome Laboratories) kindly supplied by R. O. Thomson.

Sensitization of sheep erythrocytes. Sheep blood was collected in an equal volume of Alsever solution, stored at 4°C, and used for a period of no longer than 9 days. The erythrocytes were washed three times in normal saline before sensitization, and normal saline was used as diluent for all reagents. Published methods for sensitizing erythrocytes with antibody (15, 19, 31) were tested by using twofold serial dilutions of antitoxic IgG with 5-, 10-, 15-, 20-, and 25-fold dilutions of freshly prepared 0.0375 M $CrCl_3$ stock solution in a checkerboard array. The samples of sensitized erythrocytes were then tested in reversed passive hemagglutination assays (12) with twofold dilutions of purified diphtherial toxin to determine the optimal conditions for coating the erythrocytes with antibody. Typical conditions for sensitizing erythrocytes were as follows. A 2.1-ml amount of antitoxic IgG (diluted to approximately 1 mg/ml) and 2.1 ml of a 1:20 dilution of freshly prepared 0.0375 M $CrCl_3$ were mixed with 1.05 ml of washed packed sheep erythrocytes in a sterile 50-ml polypropylene centrifuge tube and agitated gently for 5 min. The erythrocytes in each sample were then washed three times with normal saline, collected by centrifugation (3 min at 300 × *g*), and combined with 15 ml of molten agar medium at 56°C to yield 7% (vol/vol) sensitized erythrocytes, and 3-ml samples were immediately poured as overlays into petri dishes containing agar medium as described below.

Passive immune hemolysis tests. All media for this assay were prepared in acid-cleaned glassware. Deferrated agar medium (DA-1), a modification of a published medium (14), was prepared in the following manner. Proteose peptone (20 g/liter; Difco Laboratories, Detroit, Mich.) and NaCl (8.5 g/liter) were dissolved in quartz-distilled water, and the pH was adjusted to 7.4. Noble agar (10 g/liter; Difco) and Chelex 100 (10 g/liter; Bio-Rad Laboratories, Richmond, Calif.) were added, and the mixture was stirred on a magnetic stirrer at room temperature for at least 1.5 h. The medium was then sterilized by autoclaving and was subsequently cooled to 56°C in a water bath, during which time the Chelex resin settled out. The molten DA-1 medium was then decanted into a separate sterile bottle or pipetted into petri dishes without resuspending the Chelex resin. For the purposes described below, similar deferrated media were also prepared with reduced concentrations of Noble agar (DA-0.5 medium, 5 g/liter; DA-0.35 medium, 3.5 g/liter). We have noted lot-to-lot variation in the quantity of Chelex 100 resin required for preparation of satisfactory DA medium. Thus, each new lot of the resin should be tested empirically to determine the concentration required to sufficiently deferrate the DA medium.

To perform quantitative radial passive immune hemolysis assays for diphtherial toxin antigen, 60-mm plastic petri dishes were prepared, each with a basal layer containing 4 ml of DA-1 medium and an overlay containing 2 ml of DA-1 medium, 7% (vol/vol) freshly prepared antitoxin-sensitized sheep erythrocytes, and NaN_3 (2 g/liter). Wells (2-mm diameter) were punched and filled with 5-μl samples containing known quantities of purified diphtherial toxin (0.5 to 10 μg/ml), and the plates were incubated overnight at 4°C. Finally, 1-ml samples of DA-0.5 medium at 56°C were supplemented with hyperimmune goat diphtherial antiserum (final dilution, 1:50, 1:100, or 1:200) and guinea pig complement (final dilution, 1:4 or 1:8; Grand Island Biological Co., Grand Island, N.Y.) and were poured immediately into the petri dishes as overlays. The plates were then incubated at 37°C for 4 h and examined for the presence and the intensity of hemolytic zones around the wells.

The following procedures were used to detect diphtherial toxin antigen in individual plaques of corynebacteriophages. Phage stocks of known titer were combined with log-phase cultures (absorbance at 590 nm, 0.3) of *C. diphtheriae* C7 in deferrated PGT-2% maltose medium containing 0.075 μg of Fe^{2+} per ml and allowed to absorb for 20 min at 37°C under standardized conditions (11). After appropriate dilution in deferrated medium, 0.05-ml samples of the adsorption mixtures were added to tubes at 48°C containing 1-ml samples of molten DA-0.35 medium, 0.10 ml of deferrated glucose supplement (11), and 0.05 ml of the appropriate log-phase indicator cells (strain C7 at an absorbance at 590 nm of 0.3 or strain C7/β^{vir}/β^{hc} at an absorbance at 590 nm of 0.5). The samples were mixed immediately, poured as overlays into plastic petri dishes (15 by 100 mm) containing 25-ml samples of DA-1 agar, allowed to solidify at room temperature, and incubated at 37°C for 24 h to permit development of plaques. The plates were then overlaid with 3-ml samples of DA-0.5 agar containing 7% (vol/vol) sheep erythrocytes sensitized with antitoxic IgG and were incubated overnight at 4°C. Finally, an overlay with 2 ml of DA-0.5 agar containing goat antitoxic serum (1:100) and guinea pig complement (1:4) was added to each plate. The plates were then incubated for 4 h at 37°C, held overnight at room temperature, and observed for zones of immune hemolysis surrounding individual plaques.

To determine the concentration of iron required to inhibit production of diphtherial toxin in phage plaques in this assay system, appropriately diluted samples from a stock solution of $FeSO_4$ (2,000 μg of Fe^{2+} per ml) in 1 N HCl were added to the DA-1 and DA-0.35 media to achieve final concentrations of added Fe^{2+} of from 0 to 1.6 μg/ml in the phage assay media. After incubation to permit development of plaques, the passive immune hemolysis assays for diphtherial toxin antigen were completed as described above.

RESULTS AND DISCUSSION

In the experiments reported here, the technique of passive immune hemolysis (lysis of antibody-coated erythrocytes by antigen, antibody, and complement) (15, 19) was applied to detect diphtherial toxin or immunochemically related

mutant proteins (cross-reacting materials [CRMs]) in individual plaques of corynebacteriophages. The medium used for the assays was developed by empirical modifications of a previously described medium to fulfill the following criteria: (i) it had to be suitable for plaque assays of corynebacteriophages; (ii) it had to be suitable for production of diphtherial toxin during vegetative phage growth; (iii) it had to allow antitoxin-sensitized sheep erythrocytes to survive without nonspecific lysis; and (iv) it had to be suitable for detection of passive immune hemolysis. The observations summarized below were found to be most important for the development of this assay system.

In our laboratory tryptose agar is routinely used for plaque assays of corynebacteriophages (11), and modified M4 medium containing proteose peptone and horse serum is routinely used for Elek tests to detect production of diphtherial toxin (10, 14). Previous studies have established that proteose peptone is superior to tryptose in medium to be used for Elek tests (14). When plaque assays were performed with modified M4 medium, an opacity developed in the agar beneath the confluent lawns of the indicator strain of *C. diphtheriae* and obscured recognition of phage plaques. When horse serum was omitted, this opacification of the agar medium did not develop, but the medium without horse serum was unsuitable for Elek tests. We therefore investigated methods other than addition of horse serum to decrease the availability of iron in the agar medium and to maintain the suitability of the medium for production of diphtherial toxin. Addition of the iron-binding protein conalbumin (type 1; Sigma Chemical Co., St Louis, Mo.) at concentrations up to 170 μg/ml did not provide a satisfactory substitute for horse serum. However, when modified M4 medium lacking horse serum was deferrated by treatment with Chelex 100 as described above, the medium was suitable both for Elek tests and for assays of corynebacteriophages. After the concentration of added NaCl was increased to 8.5 g/liter to prevent hypotonic lysis of the sensitized erythrocytes, the reformulated medium containing 1% Noble agar was designated DA-1. For plaque assays of corynebacteriophages with this medium, reduction of the agar concentration in the overlay to 0.35% (DA-0.35 medium) and reduction of the overlay volume to 1.0 ml was found to result in larger plaques (2 to 3 mm for wild-type β^{tox+} or γ^{tox-}) and in more uniform plaque morphology.

Optimal conditions for sensitizing sheep erythrocytes with the IgG fraction from hyperimmune goat antitoxic serum in the presence of $CrCl_3$ were determined as described above. In microtiter reversed passive hemagglutination tests as little as 80 pg of diphtherial toxin could be detected, comparing favorably with the sensitivity of 20 pg/assay observed previously when tanned, Formalin-fixed sheep erythrocytes coated with equine diphtherial antitoxin purified by immunoabsorption were used in similar reversed passive hemagglutination tests (12).

In the next experiment, antitoxin-sensitized sheep erythrocytes were incorporated into DA-1 medium containing NaN_3, and the conditions required for detection of diphtherial toxin by radial passive immune hemolysis were determined by using purified diphtherial toxin and varying concentrations of goat antiserum and guinea pig complement. The optimal results were obtained with final dilutions of 1:100 for antiserum and 1:4 for complement (Fig. 1). The diameter of the hemolytic zone was found to be proportional to the logarithm of the concentration of diphtherial toxin in the test sample, and the sensitivity for diphtherial toxin was less than 1 μg/ml (5 ng/assay).

When passive immune hemolysis tests were used according to methods described above to test for diphtherial toxin antigen produced in individual plaques of corynebacteriophage β^{tox+}, large hemolytic halos developed around the plaques. Typical results are shown in Fig. 2A.

A variety of control experiments was per-

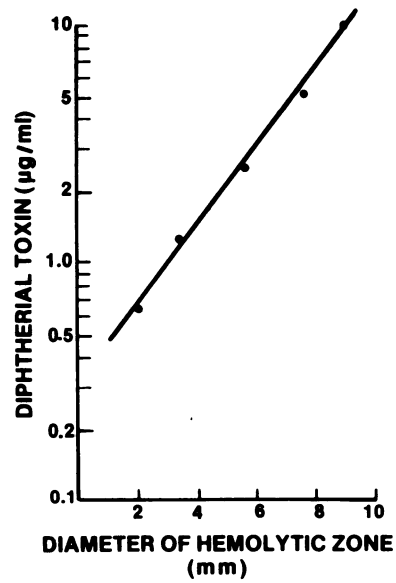

FIG. 1. *Quantitative radial passive immune hemolysis assay for diphtherial toxin. The diameter of the hemolytic halo surrounding each well was linearly related to the logarithm of the concentration of toxin in the sample added to the well. Each point represents the average of duplicate tests.*

formed to demonstrate the specificity of the hemolytic halos for diphtherial toxin or related CRMs (Table 1 and Fig. 2). Hemolytic halos were noted around plaques of the tox^+ phages β^{tox^+}, $\beta^{h_1 tox^+ h'_2}$, and $\beta^{tox^+}_{cI1}$, but no halos were observed around plaques of the tox^- CRM^- phages γ^{tox^-}, $\beta^{tox\text{-}7}$, and $\beta^{h_1 tox^- h'_2}$. Phage $\beta^{tox\text{-}197}$, which codes for a 62,000-dalton CRM, produced plaques that were surrounded by distinct halos that were smaller than those around plaques of β^{tox^+}. Since CRM197 has been shown to be immunochemically indistinguishable from diphtherial toxin (25, 30), the smaller size of the hemolytic halos indicates that less toxin antigen is present in plaques of $\beta^{tox\text{-}197}$ than in plaques of β^{tox^+} under the conditions of the passive immune hemolysis assay. This could be due either to decreased production or increased destruction of toxin antigen in plaques of $\beta^{tox\text{-}197}$ as compared with plaques of β^{tox^+}. Small and variable halos were observed around plaques of $\beta^{tox\text{-}45}$ and $\beta^{tox\text{-}30}$, which code for CRM45 and CRM30, but no halos were detected around plaques of $\beta^{tox\text{-}1}$, which codes for the smaller and more weakly cross-reacting mutant protein CRM20 (10). Thus, the development of a hemolytic halo around a corynebacteriophage plaque is controlled by the phage locus *tox* and reflects the production of diphtherial toxin or a related CRM in the phage plaque.

Several experiments were performed to determine the potential usefulness of this assay for

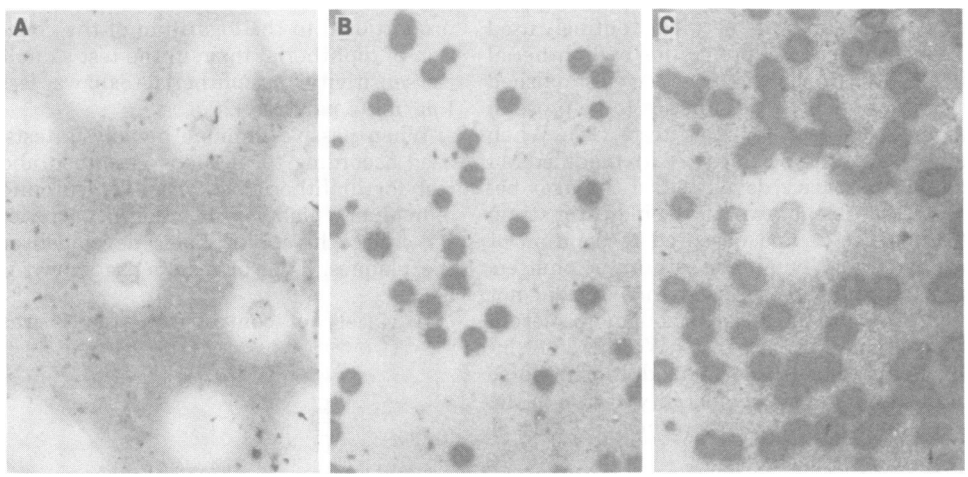

FIG. 2. *Application of radial immune hemolysis method to detect diphtherial toxin antigen produced in individual plaques of corynebacteriophages. (A) Plaques of phage β^{tox^+} in deferrated medium are surrounded by hemolytic halos. (B) Plaques of β^{tox^+} in medium supplemented with Fe^{2+} (1.6 μg/ml) are not surrounded by hemolytic halos. The plaques appear darker than the surrounding overlay due to settling of the sheep erythrocytes into the depressions created by the plaques. (C) Plaques of phages β^{tox^+} and γ^{tox^-} in deferrated medium (Table 2, footnote a). The single plaque of phage β^{tox^+} is easily identified by its large hemolytic halo among a large excess of plaques of phage γ^{tox^-}.*

TABLE 1. *Passive immune hemolysis tests with tox^+ and tox^- strains of corynebacteriophages*

Phage strain	Bacterial indicator strain	Established characteristics of phage[a]		Diam of hemolytic halo (mm)
		Mol wt of toxin or CRM	Elek test	
β^{tox^+}	C7	62,000	+	9–11
γ^{tox^-}	C7		−	None
$\beta^{tox\text{-}197}$	C7	62,000	+	4–5
$\beta^{tox\text{-}45}$	C7	45,000	+	Trace[b]
$\beta^{tox\text{-}30}$	C7	30,000	−	Trace
$\beta^{tox\text{-}1}$	C7	20,000	−	None
$\beta^{tox\text{-}7}$	C7		−	None
$\beta^{h_1 tox^+ h'_2}$	$C7/\beta^{vir}/\beta^{hc}$	62,000	+	3–7
$\beta^{h_1 tox^- h'_2}$	$C7/\beta^{vir}/\beta^{hc}$		−	None

[a] See reference 10.

[b] Reactions designated trace were small in diameter, indistinct, and variable from plaque to plaque.

genetic studies of corynebacteriophages. We demonstrated that plaques formed by the recombinant phages $\beta^{h_1 tox^+ h'_2}$ and $\beta^{h_1 tox^- h'_2}$, which were originally isolated as recombinants in matings between appropriate strains of *tox*$^+$ and *tox*$^-$ parental phages (11), could be distinguished phenotypically by the presence or absence of halos in the passive immune hemolysis test (Table 1). Furthermore, we demonstrated that plaques formed by *tox*$^+$ phages can be detected quantitatively in the presence of a large excess of plaques of *tox*$^-$ CRM$^-$ phages (Table 2 and Fig. 2C). This latter experiment indicates that the passive immune hemolysis test should be capable of detecting the rare *tox*$^+$ recombinant phages produced in matings between *tox*$^-$ CRM$^-$ parental phages carrying appropriate alleles at the *tox* locus (10). Thus, substitution of the passive immune hemolysis assay for the more time consuming assays used previously could greatly simplify the analysis of *tox* genotypes in recombinant phages in genetic studies, provided of course that the parental or recombinant *tox* genotypes can be accurately inferred from tests for toxin antigen.

It is well known that excess iron in cultures of *C. diphtheriae* reduces the yield of diphtherial toxin (1, 3, 23, 24), and phage $\beta^{tox^+}_{cI1}$ was isolated as a phage mutant that is partially insensitive to this effect of iron on toxinogenesis (21). To investigate the effects of iron under the conditions of the present assay system, DA-1 and DA-0.35 media were supplemented with Fe^{2+} at concentrations up to 1.6 μg/ml, and plaques of phages β^{tox^+} and $\beta^{tox^+}_{cI1}$ were compared by passive immune hemolysis tests in the iron-supplemented media (Fig. 2 and Table 3). Although plaques of $\beta^{tox^+}_{cI1}$ were surrounded by slightly larger halos than plaques of β^{tox^+} at concentrations of Fe^{2+} between 0.1 and 0.4 μg/ml, plaques of both phage strains lacked halos at Fe^{2+} concentrations of 0.8 μg/ml or greater. Additional control experiments demonstrated that concentrations of Fe^{2+} up to 1.6 μg/ml did not interfere with the detection of purified diphtherial toxin in radial passive immune hemolysis assays similar to those shown in Fig. 1. Since *tox*$^+$ phages can be easily detected among a large excess of *tox*$^-$ phages by the immune hemolysis assay (Table 2 and Fig. 2C), it is possible to screen large numbers of phage plaques for rare mutants that produce hemolytic halos in iron-supplemented medium. By this method we have recently isolated a series of independent mutants of phage β^{tox^+}, and we have shown that C7 lysogens harboring these mutant phages produce large amounts of diphtherial toxin antigen under high-iron conditions (unpublished data). These newly isolated phage mutants will be described in detail in a subsequent publication.

TABLE 2. *Detection of rare tox*$^+$ *phages in a population of tox*$^-$ *CRM*$^-$ *corynebacteriophages*[a]

Dilution of phage inoculum	Avg plaque counts[b]	
	Total	*tox*$^+$
1	Confluent lysis	205
10^{-1}	Nearly confluent lysis	15.3
10^{-2}	540	1.5
10^{-3}	55.5	0

[a] Stocks of phages β^{tox^+} and γ^{tox^-} were mixed to produce a ratio of approximately 300 *tox*$^-$ phages per *tox*$^+$ phage. The mixture was assayed for infectivity, and the plaques were tested for toxin antigen by the passive immune hemolysis test as described in the text.

[b] From two or more plates.

TABLE 3. *Effect of excess iron on production of diphtherial toxin in individual plaques of corynebacteriophages*

Concn of added Fe^{2+} (μg/ml)	Diam (mm) of hemolytic halo	
	C7(β^{tox^+})	C7($\beta^{tox^+}_{cI1}$)
0	9–10	9–10
0.1	3–4	5–6
0.2	Trace[a]	Trace
0.4	None	Trace
0.8	None	None
1.6	None	None

[a] Reactions designated trace were small in diameter, indistinct, and variable from plaque to plaque.

A previous study from our laboratory demonstrated that passive immune hemolysis tests can be used to detect heat-labile enterotoxin produced by individual colonies of *Vibrio cholerae* or *Escherichia coli* (2). The assay for diphtherial toxin described here is different in several respects. Although the heat-labile enterotoxins of *V. cholerae* and *E. coli* bind spontaneously to receptors on sheep erythrocytes and sensitize them to immune hemolysis, control experiments demonstrated that diphtherial toxin does not spontaneously sensitize sheep erythrocytes. Thus, it was necessary to use antitoxin-sensitized erythrocytes in the present study. When specific passive immune hemolysis tests are used to detect antigens produced by bacterial colonies, the relevant antigens must be available to interact with the sensitized erythrocytes. Thus, unless the relevant antigens are extracellular products or are released from bacterial colonies by cell lysis, it may be impossible to detect them by the passive immune hemolysis

method. In contrast, extensive bacterial lysis does occur during the development of bacteriophage plaques, and passive immune hemolysis techniques of the type described here could theoretically be used to detect any antigen released during lysis of phage-infected cells. Practical limitations to be considered in applying this technology include the needs for relatively large quantities of specific antibodies, for production or release of detectable quantities of specific antigens in the phage plaques, for the absence of interfering hemolytic substances, and for medium that is not anticomplementary.

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