Detection and Identification of Diagnostic 
Histoplasma capsulatum Precipitates by 
Counterelectrophoresis

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Studies were carried out to develop and evaluate a counterelectrophoresis (CEP) technique for the rapid and specific identification of the diagnostically important histoplasmosis H and M precipitin bands. Well-defined and centrally located precipitin bands were produced by using a discontinuous buffer system and a gel matrix composed of agarose and ion agar no. 2. A template was devised which allowed the selective identification of the H and M precipitins. Comparative evaluations were performed with the microimmunodiffusion (ID) and complement fixation tests. In 52 sera from persons with histoplasmosis, either the H or M precipitin, or both, were identified in 42 (81%) of the cases with the CEP technique and in 43 (83%) with the ID test. With sera from 28 persons with heterologous diseases, the CEP technique, like the ID test, failed to react. The specificity of the CEP technique was dependent upon the use of the identity template. The CEP technique is recommended for routine use in laboratories testing moderate numbers of sera. It provides accurate and reproducible results within 90 min, in contrast to the ID test, which requires 18 to 24 h.

Counterelectrophoresis (CEP) provides the laboratory with a rapid means of detecting antigens and antibodies (1, 2, 3, 5, 8, 12). Although its greatest use has been in the detection of Australia antigen, it has also been applied recently to the serodiagnosis of systemic mycotic infections (4, 14).

In 1971, we initiated a study to determine the applicability of the CEP technique to the serodiagnosis of histoplasmosis. Our goals were threefold: (i) to define the optimal conditions for the rapid formation of centrally located, well-defined, or distinct H and M precipitin bands, (ii) to design an identity template that would permit the specific identification of the diagnostically important H and M precipitin bands, and (iii) to compare the sensitivity and specificity of the optimal CEP technique incorporating the selected identity template with that of the conventional complement fixation (CF) and immunodiffusion (ID) tests.

During this investigation, Gordon et al. (4) reported on the application of the CEP technique to the serodiagnosis of histoplasmosis and other mycotic infections. By using sera from eight persons suspected of having histoplasmosis, Gordon and his co-workers demonstrated the potential of the technique to rapidly detect Histoplasma capsulatum precipitins.

The development and evaluation of a CEP technique that permits the simultaneous detection and identification of centrally located and well-defined H and M precipitin bands are described in this paper.

”This is part of a dissertation submitted by B.K. in partial fulfillment of the requirements for the Ph.D. degree at the University of North Carolina.”

MATERIALS AND METHODS

CF and ID tests. CF tests were performed according to previously published procedures (13) by using Blastomyces dermatitidis ground yeast-form antigen (Center for Disease Control [CDC] lot 24), coccidiodin (CDC lot 1), H. capsulatum whole yeast cells (CDC lot 45), and histoplasmin (CDC lot 13). ID tests were performed according to published procedures (10) with B. dermatitidis yeast-form filtrate antigen (CDC lot 10), coccidiodin (CDC lot 1), and histoplasmin (CDC lot 8). This lot of histoplasmin was prepared by pooling the mycelial culture filtrates of eight isolates of H. capsulatum grown individually for 6 months at 25°C in Smith asparagine medium.
Thimerosol was added to a final concentration of 1:5,000. After 1 week at 25 C, the H. capsulatum filtrate pool was filtered through a membrane filter (0.45 μm pore size; Millipore Corp.) and then concentrated to one-tenth of its original volume by use of an Amicon ultrafiltration system. This lot of histoplasmin was also used in the CEP test.

Sera. Sera from patients with culturally proven cases of aspergillosis, blastomycosis, coccidiodomycosis, and histoplasmosis were obtained from the Fungus Immunology Section's serum collection at the CDC. The control serum (CDC lot 39) was obtained from the CDC's Biological Reagents Section.

Counterelectrophoresis test. Projector slide cover glasses (3.25 by 4 in; approximately 8.2 by 10 cm) were precoated with molten 1% agarose (Industrie Biologique Francaise S. A.) in distilled water by using a cotton swab and dried at 37 C. A portion (10 ml) of an equal mixture of 0.85% agarose and 0.85% ionagar no. 2, dissolved in 0.01 M Veronal buffer, pH 7.2, was then applied. If necessary, plates can be stored in a humidity chamber (Grafar, Detroit, Mich.) at room temperature for 1 week. A 75-well pattern, consisting of three parallel columns of paired wells, was cut (Fig. 1). Each column contained 13 serum wells and 12 antigen wells. Wells were 5 mm in diameter. Serum wells were separated by 1 mm from edge to edge, as were the antigen wells. Each antigen well was 3 mm from each of two serum wells. The wells were cut through a plastic template with a beveled metal borer. The agar plugs were removed by suction.

Serum to be tested for antibody was placed in the anodic wells of each pair, and histoplasmin was placed in the cathodic wells. A control serum containing H and M antibodies was placed in the wells adjacent to the serum to be tested. Wells were filled once with capillary hematocrit tubes to the level of the agar (0.025 ml). Electrophoresis was performed in a model EP-1 Meloy electrophoresis unit (Meloy Laboratories, Springfield, Va.) with 0.05 M Veronal buffer, pH 7.2, in each chamber. Telfa wicks (Telfa Non-Adherent Strips, 2.5 by 4 in, Kendall Hospital Products Div., Chicago, Ill.) were used to connect the slides to the buffer. A constant current of 25 mA was applied across the narrow dimension of the slide for 90 min. Electrophoresis was performed at room temperature. Slides were read against a dark background with indirect light. The results were recorded and the slides were stained with thiazine red R, according to the procedure of Palmer et al. (11). No reactions were seen in the stained slide that had not been observed in the unstained slide; however, stained slides were more easily photographed.

RESULTS

Optimal test conditions. Preliminary studies with the combination of variables most widely used in the detection of Australia antigen—0.85% agarose prepared in 0.05 M Veronal buffer, pH 8.2, with the same buffer used in the electrode chambers—did not permit the development of well-defined and centrally located histoplasmosis precipitins. The M band was diffuse, and the H band was just adjacent to, or even overlapped, the serum well.

Studies were therefore carried out to determine the optimal conditions necessary to achieve our first objective, the formation of centrally located and well-defined H and M precipitin bands. The variables tested are outlined in Table 1. Agarose, at a concentration of 0.85%, was dissolved in one of four buffers. The buffer used in the electrode chambers was 0.05 M Veronal, pH 7.2, in all instances. Well-defined H and M bands, with the H band overlapping the serum well, were formed in agarose dissolved in 0.01 M Veronal buffer.

Slides that had been precoated with 1% agarose in distilled water were coated with 10 ml of any one or a combination of several agars at a concentration of 0.85% in 0.01 M Veronal buffer, pH 7.2. In all instances, 0.05 M Veronal buffer, pH 7.2, was used in the electrode chambers. An equal mixture of agarose and ionagar no. 2 resulted in centered, intense precipitin bands, and the number of precipitin bands was consistent with the number expected.

An equal mixture of agarose and ionagar no. 2 was dissolved in 0.01 M Veronal buffer, pH 7.2, and one of three buffers was used in the electrode chambers (Table 1). The use of 0.05 M Veronal buffer, pH 7.2, in the electrode chambers resulted in well-centered, intense, and well-defined precipitin bands. Results with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.9, were comparable with those obtained with 0.05 M Veronal, pH 7.2, but the use of two different buffer systems, Veronal and Tris, added a degree of complexity that was deemed undesirable.

FIG. 1. Counter-electrophoresis identity template. Wells are 5 mm in diameter. Serum wells are 1 mm from edge to edge, as are antigen wells. Every antigen well is 3 mm from each of two serum wells. ×0.33.
Table 1. Variables of the histoplasmosis counterelectrophoresis test and their effect upon the position, intensity and definition of the precipitin bands

| Variable*                              | Centered | Intensity | Definition         |
|----------------------------------------|----------|-----------|--------------------|
| Buffer used for suspension of agarose (0.85%) |          |           |                    |
| 0.01 M Veronal, pH 7.2                 | No       | Strong    | Good               |
| 0.05 M Veronal, pH 7.2                 | No       | Moderate  | Fair               |
| 0.05 M Veronal, pH 8.2                 | No       | Moderate  | Fair               |
| 0.01 M Tris, pH 8.9                    | No       | Moderate  | Poor               |
| Agar types dissolved in 0.01 M Veronal, pH 7.2 |          |           |                    |
| Agar, agar-agar, or Noble agar         | No       | Weak      | No M band          |
| Ionagar no. 2                          | No       | Strong    | H and M bands      |
| Agarose                                | No       | Strong    | H and M bands      |
| Equal mixture of agarose and Noble agar| No       | Strong    | No M band          |
| Equal mixture of agarose and ionagar No. 2 | Yes     | Strong    | H and M bands      |
| Buffer used in electrode chambers with 0.01 M Veronal used to suspend an equal mixture of agarose and ionagar no. 2 | | | |
| 0.05 M Veronal, pH 7.2                 | Yes      | Strong    | Good               |
| 0.05 M Veronal, pH 8.2                 | Yes      | Weak      | Poor               |
| 0.01 M Tris, pH 8.9                    | Yes      | Moderate  | Good               |
| Time (min)                             |          |           |                    |
| 60                                     | Yes      | Moderate  | No identity pattern for H band |
| 90 and 120                             | Yes      | Strong    | Identity pattern for H band |

*Variable of choice is in italics.

Electrophoresis for 60 min resulted in well-centered, moderately intense, but ill-defined H and M precipitin bands. After 90 min of electrophoresis, the H and M precipitin bands were well-centered, intense, and well-defined. Electrophoresis for 120 min did not further intensify precipitin formation.

Development of an identity template. Only two of the six precipitating antibodies to histoplasmin described by Heiner (6), the H and M antibodies, are considered to be of diagnostic importance. Consequently, one of the objectives of this study was to develop a template that would permit the specific identification of the precipitin bands formed by the interaction of H and M antibodies with histoplasmin and their differentiation from precipitin bands resulting from the interaction of other antibodies with histoplasmin.

When a template was designed so that the serum wells were 3 mm apart and the antigen well was equidistant (5 mm) from two serum wells, high electrophoretic mobility of the H antigen seemed to cause it to migrate between the serum wells, resulting in minimal interaction with the H antibody. Therefore, we decided to place the serum wells as close together as possible to obtain maximal interaction between antigen and antibody.

The template that was designed is shown in Fig. 1. Serum wells 5 mm in diameter were placed 1 mm apart and antigen wells 5 mm in diameter were placed so that they were 3 mm from each of two serum wells. Results obtained by use of this template with control serum containing H and M antibodies and test sera placed in alternate wells are shown in Fig. 2. Preliminary evaluations with this identity template indicated the procedure to be of potential diagnostic value. The results were very similar to those obtained by use of the ID test. Sera with M antibody, as determined with the ID test, produced a line of identity with the M precipitin band when reacted with histoplasmin in the CEP technique; sera with both H and M antibodies produced two lines of identity with the control serum containing H and M antibodies, and sera from patients with culturally proven histoplasmosis that were negative in the ID test were negative or produced lines of nonidentity with either the H or M precipitin lines (Fig. 2, wells no. 23, 25, 30). The diagnostic importance of these unidentified lines observed with the CEP technique remains undetermined.

High magnifications of the patterns of identity and nonidentity are shown in Fig. 3 and 4. Reactions of identity for both the H and M precipitin bands were clearly visible and dis-
Fig. 2. Reactions of identity by counterelectrophoresis with control serum known to contain H and M antibodies in alternating wells 1-13, 14-26, and 27-39, and sera from persons with culturally proven histoplasmosis (×1.3). Stained with thiazine red R. Ag, histoplasmin wells; serum, serum wells. Wells no. 2, 10, and 12 contained serum having only the M antibody as determined with the ID test; wells no. 4, 6, and 8 contained serum having both the H and M antibody. Wells no. 15, 17, 19, and 21 contained serum having only the M antibody as determined by the ID test. Wells no. 23 and 25 contained sera that demonstrated no precipitin lines in the ID test. The precipitin line seen in the counterelectrophoresis technique with these sera (no. 23 and 25) was not identical with either the H or M precipitin lines. Wells no. 28, 32, 34, and 36 contained serum having only the M antibody as determined by the ID test. Well no. 30 contained serum that was negative in the ID test; once again, a line of nonidentity was produced. Well no. 38 contained serum having both the H and M antibodies, as determined in the ID test.

Fig. 3. Reactions of identity by counterelectrophoresis with control serum known to contain H and M antibodies (wells 2, 4, and 6) and sera from persons with culturally proven histoplasmosis (wells 1, 3 and 5). Stained with thiazine red R.

pliment-fixing antibodies in 46 (88%) of the 52 sera. Five of the sera were anticomplementary and were considered negative. Note the extreme cross-reactivity of the histoplasmosis case sera in the CF test; 28 (54%) of 52 of the sera from persons with proven histoplasmosis reacted with the B. dermatitidis antigen. Some of the heterologous titers were identical with or higher than the homologous titers.

In the ID test with histoplasmin antigen, antibodies were detected in 43 (83%) of 52 cases, and in the CEP test, antibodies were detected in 42 (81%) of the 52 histoplasmosis cases. A serum was considered positive when it contained a precipitin band identical with that of the H or M precipitins, or both (Fig. 2). None of the 52 histoplasmosis case sera reacted in ID tests with B. dermatitidis or coccidioidin precipitinogens. In one instance (serum 20), the CEP test
tinct (Fig. 3). Figure 4 illustrates both a reaction of identity for the M precipitin and a reaction of nonidentity.

Sensitivity of the CEP test. The sensitivity of the CEP test was compared to that of the conventional tests most widely used in the serodiagnosis of histoplasmosis with sera from 52 persons with proven histoplasmosis (Table 2). The CF test, with histoplasmin and yeast-form antigens of H. capsulatum, detected com-
from persons with aspergillosis (A1 and A3) reacted with histoplasmin in the CEP test to produce lines of nonidentity. These lines are of unknown diagnostic value, and the sera were considered negative.

**DISCUSSION**

Gordon et al. (4) reported the development of a CEP technique for histoplasmosis. Their CEP technique was basically similar to the one used by Prince and Burke (12) for the detection of Australia antigen. In this technique, a continuous buffer system (0.05 M Veronal, pH 8.8) and a matrix composed solely of agarose were used.

A study of the factors affecting the CEP technique revealed that the combination of factors optimal for the detection of Australia antigen (3, 11, 12, 15) was not optimal for the serodiagnosis of histoplasmosis.

Our study (Table 1) demonstrated that an effective CEP test required the use of a buffer system discontinuous with respect to molarity—0.05 M Veronal, pH 7.2 to 7.4, used in the electrode chambers and 0.01 M Veronal, pH 7.2 to 7.4, used to suspend the matrix. The discontinuous buffer system was complemented by use of a matrix composed of equal parts of 0.85% agar and ionagar no. 2 and by electrophoresis for 90 min. This optimal combination of factors allowed the formation of well-defined, intense, and well-centered H and M precipitins.

Alter et al. (1) described a CEP test template for the identification of the precipitates formed by the interaction of Australia antigen and antibody. However, this development has remained relatively unnoticed. In a recent publication, Schmidt and Lennette (16) stated that the ID test must be used to confirm the identity of positive reactions obtained with the CEP test. This was necessary because the CEP test without an identity pattern may produce false-positive reactions (1). A template which permits the identification of precipitates formed with the CEP technique allows the simultaneous detection and identification of precipitating antigen-antibody.

The need for reference precipitin bands to determine lines of identity in the ID test is well established in the serodiagnosis of mycotic infections. Although Gordon et al. (4) demonstrated lines of identity for the M precipitin bands, they did not succeed in demonstrating an identity pattern for the H precipitin bands. In contrast, the CEP technique and identity template developed in this study permit the demonstration of lines of identity for both the H and M precipitin bands (Fig. 1 and Table 1).

**Fig. 4. Reactions of identity and nonidentity by counterelectrophoresis with control serum known to contain H and M antibodies (wells 1, 3, and 5) and sera from persons with culturally proven histoplasmosis. x3.4. Stained with thiazine red R. The diffuse precipitin band formed by the serum in well 4 was unrelated to either the H or M precipitin bands.**

**Specificity of the CEP test.** Sera from persons with proven mycotic infections other than histoplasmosis and from normal persons were tested in the CF test with histoplasmin and *H. capsulatum* yeast-form antigens and in the ID and CEP tests with histoplasmin. The data in Table 2 again indicate the extremely low specificity of the CF test. Eighteen (65%) of 28 sera from persons with diseases other than histoplasmosis reacted in the CF test with the *H. capsulatum* antigens. In contrast, none of the heterologous or normal sera demonstrated the diagnostically important H or M precipitins in the CEP or ID tests. However, one serum from a person with cryptococcosis (Cr 1) and two sera
Contrary to our expectations, there was little difference in sensitivity between the ID and CEP tests in detecting precipitating *H. capsulatum* antibodies in sera from 52 persons with proven histoplasmosis.

The lack of increased sensitivity of the CEP test for *H. capsulatum* antibodies, when compared with the ID test, was surprising in the light of the work that has been done with Australia antigen. In studies with Australia antigen, Holper and Jambazian (7) found the CEP test to be eight times more sensitive than the ID test, Schmidt et al. (15) found it four- to sixteenfold more sensitive, and Alter et al. (1) found it 21 times more sensitive. Theoretically, one might expect the CEP test to be more sensitive than the ID test, since in the former the reactants are forced toward each other in one direction and in the latter the reactants merely diffuse radially in all directions from the point of origin. However, the lack of increased sensitivity of the CEP test in the serodiagnosis of histoplasmosis, when compared with the ID test, is in agreement with the findings of Gordon et al. (4). These workers found that two sera from suspected cases of histoplasmosis, which had CF titers of 1:4 and 1:16 with the *H. capsulatum* yeast-form antigen and were negative in the ID test, were also negative by the CEP test.

In accord with previous observations (9), the results of this study confirm the high degree of nonspecificity of the CF test for histoplasmosis. In contrast to the CF test, the CEP test, like the ID test, was 100% specific. Demonstration of the specificity of the CEP test was dependent on the use of the identity template with a control serum. That such a control was mandatory for specific diagnosis of histoplasmosis was indicated by the fact that three sera from persons with mycotic diseases other than histoplasmosis produced precipitin bands not identical with either the H or M precipitin bands.

The CEP test for the serodiagnosis of histo-

**Table 2. Reactions of sera from 52 persons with proven histoplasmosis in CF, ID, and CEP tests**

| Serum no. | CF titer* | ID bands* | CEP bands* | Serum no. | CF titer* | ID bands* | CEP bands* |
|-----------|-----------|-----------|------------|-----------|-----------|-----------|------------|
|           | Hi Y B C  | Hi Hi     |            |           | Hi Y B C  | Hi Hi     |            |
| 1         | 8 32 0 0  | M M       |            | 27        | 0 16 8 0  | M M       |            |
| 2         | 64 64 0 0 | M M       |            | 28        | 256 128 0 | M M       |            |
| 3         | 32 64 0 0 | M M       |            | 29        | 64 64 32 0| M M       |            |
| 4         | 0 32 0 0  | M M       |            | 30        | 32 32 8 0 | M M       |            |
| 5         | 0 32 0 0  | M M       |            | 31        | 8 16 8 0  | M M       |            |
| 6         | 0 8 0 0   | M M       |            | 32        | 32 128 16 | M M       |            |
| 7         | 0 8 16 0  | M M       |            | 33        | 32 8 0 0  | H H       |            |
| 8         | 0 8 8 0   | M M       |            | 34        | 16 32 64 32 | 0 0     |            |
| 9         | 32 128 8 0 | H/M H/M  |            | 35        | 8 256 8 0 | M M       |            |
| 10        | 64 128 0 0 | M M       |            | 36        | 8 32 0 0  | M M       |            |
| 11        | 32 64 0 0 | H/M H/M  |            | 37        | 32 128 0 8 | H/M H/M |            |
| 12        | 32 32 0 0 | H/M H/M  |            | 38        | 8 16 0 0  | M M       |            |
| 13        | 32 64 32 8 M M |            | 39        | 32 32 0 0 | H/M H/M |            |
| 14        | 64 64 32 8 H/M H/M |            | 40        | 64 128 256 0 M M |            |
| 15        | 0 16 0 0  | M M       |            | 41        | 8 16 0 0  | M M       |            |
| 16        | 128 256 0 0 | M M       |            | 42        | 0 16 0 0  | M M       |            |
| 17        | 128 512 8 0 | M M       |            | 43        | 0 16 8 0  | M M       |            |
| 18        | 0 8 0 0   | M M       |            | 44        | AC 0 0   | M M       |            |
| 19        | 0 8 0 0   | M M       |            | 45        | 8 16 32 0 | 0 0       |            |
| 20        | AC 0 0    | M M       |            | 46        | 8 16 0 0  | 0 0       |            |
| 21        | AC 0 0    | M M       |            | 47        | AC 0 0   | M M       |            |
| 22        | 0 32 16 0 0 0 *  |            | 48        | 8 16 8 0  | 0 0       |            |
| 23        | 0 16 8 0 0 0 *  |            | 49        | 32 8 0 0  | M M       |            |
| 24        | 32 32 0 0 0 0 *  |            | 50        | AC 0 0   | M M       |            |
| 25        | 0 0 0 0   | 0 0 0 0 0 *  |            | 51        | 16 0 0 0 0 0 0 | M M       |            |
| 26        | 512 256 32 0 0 0 0 0 0 0 0 0 0 | H/M H/M |            | 52        | 8 32 8 0 0 0 0 0 0 | M M       |            |

*Abbreviations: Hi, histoplasmin; Y, Yeast form of *H. capsulatum*; B, *B. dermatitidis* yeast-form antigen; C, coccidioidin; AC, anticomplementary.*

*None of the sera tested were positive in the ID test with *B. dermatitidis* or *Coccidioides immitis* antigens.

*0, Negative reaction; 0*, precipitin band observed but not identical with H or M.
Blastomycosis
Aspergillosis
Cryptococcosis

and plasmosis with the encountered ID and in c0*, Precipitin Abbreviations: 0, testing 26, test from five specificity Negative M. Reactions CEP of with reactions. Serum B3 B4 B5 B6 B7 B8 A1 A2 A3 Cr1 Cr2 Cr3 Cr4 C5 C6 C7 C8 C9 C10 C11 C12 C13 N1 to N5

| Clinical category | Serum no. | CF titer* | ID bands* | CEP bands |
|-------------------|-----------|-----------|-----------|-----------|
| Blastomycosis     | B1        | 0         | 0         | 0         |
|                   | B2        | 0         | 0         | 0         |
|                   | B3        | 0         | 0         | 0         |
|                   | B4        | 0         | 0         | 0         |
|                   | B5        | 0         | 0         | 0         |
|                   | B6        | 8         | 8         | 0         |
|                   | B7        | 0         | 0         | 0         |
|                   | B8        | 0         | 0         | 0         |
| Aspergillosis     | A1        | 0         | 16        | 0         |
|                   | A2        | 8         | 16        | 0         |
|                   | A3        | 0         | 8         | 0         |
| Cryptococcosis    | Cr1       | 0         | 8         | 0         |
|                   | Cr2       | 8         | 8         | 0         |
|                   | Cr3       | 0         | 8         | 0         |
|                   | Cr4       | 0         | 0         | 0         |
| Coccidioidomyco-
|sis | C1        | 0         | 32        | 0         |
|                   | C2        | 0         | 0         | 0         |
|                   | C3        | 0         | 0         | 0         |
|                   | C4        | 0         | 0         | 0         |
|                   | C5        | 0         | 8         | 0         |
|                   | C6        | 8         | 8         | 0         |
|                   | C7        | 32        | 32        | 0         |
|                   | C8        | 16        | 32        | 0         |
|                   | C9        | 32        | 32        | 0         |
|                   | C10       | 0         | 0         | 0         |
|                   | C11       | 8         | 8         | 0         |
|                   | C12       | 0         | 0         | 0         |
|                   | C13       | 8         | 32        | 0         |
| Normal            | N1 to N5  | 0         | 0         | 0         |

* Abbreviations: Hi, histoplasmin; Y, yeast form of H. capsulatum.
* 0, Negative reactions.
* 0*, Precipitin band observed but not identical with H or M.

plasmosis combines the simplicity, sensitivity, and specificity of the ID test with the added dimension of rapidity. Results are obtained with the CEP test within 90 min, whereas the ID test takes 18 h or longer. The CEP test for histoplasmosis is a valuable adjunctive procedure in interpreting the cross-reactions so often encountered with the CF test and is also useful in testing anticomplementary sera. Use of the CF test combined with the CEP test can contribute greatly to the accurate and rapid diagnosis of histoplasmosis and to its proper treatment.

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