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# Comparison of passive haemagglutination and enzyme-linked immunosorbent assay for serodiagnosis of plague*

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*Sera of 42 suspect plague cases from Ovamboland, Namibia, were examined. ELISA proved more sensitive than passive haemagglutination for the detection of F1 antibody and increased positive serodiagnoses of plague by 7-42%.*

The enzyme-linked immunosorbent assay (ELISA) has been investigated as a method for detecting plague antibodies in man, carnivores, and rodents (*1*, *2*). Results indicate that ELISA might be an alternative to the standard passive haemagglutination test (PHA) for the diagnosis of human plague and for plague surveillance and control activities. Unlike PHA, ELISA can be used to measure specific immunoglobulins (IgG, IgM, etc.) in patients' sera or other specimens. In this paper, additional advantages of ELISA over PHA for serodiagnosis of plague are described, and the merits and limitations of these techniques are compared.

## MATERIALS AND METHODS

Human sera from the Ovamboland plague focus in Namibia (*3*, *4*) were examined for specific plague antibodies. Paired sera collected during 1976-78 from 30 patients were tested by PHA, within two weeks of sampling, and then stored at −20 °C until November-December 1980, when the sera were examined by ELISA. In addition, single sera collected in October-November 1980, from 12 cases presumptively diagnosed as plague on the basis of clinical symptoms, were tested.

The PHA test used (*3*, *5*) during 1976-78 and in 1980 was essentially the procedure currently recommended by WHO for serodiagnosis of plague (*6*, *7*). A series of twofold serum dilutions, from 1:4, were prepared from 25 µl of serum, and tested. The titre was taken to be the highest dilution exhibiting complete haemagglutination.

For ELISA, the reagents were prepared as described by Voller et al (*8*). Each well of the ELISA substrate plates[a] was sensitized with 1 µg of the fraction 1 (F1) antigen of *Yersinia pseudotuberculosis* subsp. *pestis* (previously named *Yersinia pestis*) in 100 µl of pH 9.6 coating buffer. Plates were incubated at 37 °C until dry. Excess antigen was removed by washing four times with phosphate-buffered saline containing 0.5 ml of Tween 20 per litre (PBST). Using 25 µl of serum, twofold dilutions (50 µl/well), from 1:4, were prepared with PBST directly in the substrate plate using a multichannel pipettor, and incubated for 1 hour at 37 °C. After four washes with PBST, 50 µl of a 1:150 dilution of horseradish peroxidase-conjugated immunoglobulin were added to each well and incubated for 1 hour at 37 °C. All sera were tested with anti-human IgG + IgM + IgA.[b] Sera collected in 1980 were also tested with anti-human IgG (γ-chain specific)[c] and with anti-human IgM (µ-chain specific).[d] After incubating with conjugate and another four washes with PBST, 150 µl of freshly prepared indicator solution (9 parts of 0.8 g/litre 5-aminosalicylic acid, pH 6.0, and 1 part of 0.05 ml/litre $H_2O_2$) were added to each well

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[a] M129A flat bottom Microelisa plates, Dynatech Laboratories, Inc., Alexandria, VA, USA.

[b] Code No. 61-231-1 Ly, Miles Laboratories, Inc., Elkhart, IN 46515, USA.

[c] Code No. 61-130-1, Miles Laboratories, Inc., Elkhart, IN 46515, USA.

[d] Code No. 61-132-1, Miles Laboratories, Inc., Elkhart, IN 46515, USA.

and the plates were incubated for 1 hour at 37 °C. In recording the results, the titre was taken to be the highest serum dilution producing an obvious colour reaction. Positive sera were retested to confirm the titre. The specificity of ELISA was determined by performing an ELISA-inhibition test (EI) at the same time as a positive serum was retested by ELISA. The EI differed from ELISA only in that the PBST used for preparing serum dilutions contained 100 μg of F1 antigen/ml. An ELISA titre was considered to be a measure of specific F1 antibody if the EI endpoint was at least eight times lower than the ELISA value.

A fourfold or greater increase in titre to 1:32 or more was accepted as *prima facie* evidence for a diagnosis of plague. However, owing to transport difficulties in Ovamboland, patients sometimes arrived at the hospital many days after the onset of symptoms and early rises in titre may have been missed. Consequently, a titre of 1:32 or more concurrent with typical symptoms and no history of plague vaccination were considered sufficient evidence for a positive diagnosis.

## RESULTS

ELISA gave results that were very similar to those obtained with PHA during 1976–78 (Table 1). The titres given by the two techniques were similar for most sera, and retests of positive sera demonstrated a normal test error for both PHA and ELISA of ± 1 twofold dilution. However, ELISA results for sera with titres of 1:4–1:16 were somewhat more variable

Table 1. Serological tests of human sera collected in Ovamboland during 1976–78

| Serodiagnosis of plague | Age and sex | Date of first serum | Days to second serum | Change in titre[a] | |
|---|---|---|---|---|---|
| | | | | PHA | ELISA (IgG + IgM + IgA) |
| 9 patients (30%) – negative by PHA and ELISA | 8F | 12 Oct. 77 | 5 | both < 1:4 | both < 1:4 |
| | 4M | 20 Oct. 77 | 4 | both < 1:4 | both < 1:4 |
| | 4F | 20 Oct. 77 | 5 | both < 1:4 | both < 1:4 |
| | 11F | 24 Oct. 77 | 10 | both < 1:4 | both < 1:4 |
| | 14M | 31 Oct. 77 | 4 | < 1:4 → 1:16 | < 1:4 → 1:8 |
| | 7M | 14 Nov. 77 | 3 | non-specific | both < 1:4 |
| | 8M | 9 Dec. 77 | 3 | both < 1:4 | both < 1:4 |
| | 13F | 2 Oct. 78 | 3 | both < 1:4 | both < 1:4 |
| | F[b] | 3 Nov. 78 | 7 | < 1:4 → 1:16 | both < 1:4 |
| 19 patients (63%) – positive by PHA and ELISA | 6F | 9 Oct. 76 | 3 | 1:4 → 1:16 | 1:16 → 1:8192 |
| | 8F | 3 Nov. 76 | 5 | 1:8 → 1:128 | <1:4 → 1:32 |
| | 12F | 12 Sep. 77 | 3 | <1:4 → 1:256 | <1:4 → 1:64 |
| | 12M | 20 Sep. 77 | 34 | <1:4 → 1:512 | <1:4 → 1:512 |
| | 16F[c] | 13 Oct. 77 | 11 | <1:4 → 1:64 | <1:4 → 1:256 |
| | 9F | 20 Oct. 77 | 4 | 1:16 → 1:128 | <1:4 → 1:16 |
| | 10M | 20 Oct. 77 | 5 | <1:4 → 1:128 | <1:4 → 1:64 |
| | 35F | 24 Oct. 77 | 7 | 1:512 → 1:256 | both 1:128 |
| | 37M | 24 Oct. 77 | 7 | 1:128 → 1:64 | both 1:64 |
| | 11M | 25 Oct. 77 | 9 | <1:4 → 1:64 | <1:4 → 1:32 |
| | 13F | 4 Nov. 77 | 6 | <1:4 → 1:32 | <1:4 → 1:32 |
| | 16F | 14 Dec. 77 | 9 | <1:4 → 1:128 | <1:4 → 1:128 |
| | 13F | 2 Oct. 78 | 7 | 1:8 → 1:128 | 1:4 → 1:32 |
| | 16F[c] | 3 Oct. 78 | 6 | 1:8 → 1:64 | 1:4 → 1:32 |
| | 21F | 3 Oct. 78 | 9 | <1:4 → 1:64 | <1:4 → 1:256 |
| | 14F | 4 Oct. 78 | 5 | 1:8 → 1:256 | <1:4 → 1:128 |
| | 5F | 31 Oct. 78 | 16 | <1:4 → 1:256 | <1:4 → 1:512 |
| | 8F | 16 Nov. 78 | 4 | both 1:256 | 1:256 → 1:2048 |
| | 44F | 16 Nov. 78 | 4 | both 1:128 | both 1:512 |
| 2 patients (7%) – positive by ELISA; negative by PHA | 22M | 2 Oct. 78 | 7 | 1:8 → < 1:4 | 1:32 → 1:256 |
| | 14M | 5 Oct. 78 | 14 | 1:4 → < 1:4 | <1:4 → 1:1024 |

[a] Antibody to the specific F1 antigen of *Y. pseudotuberculosis* subsp. *pestis*. PHA was done within 1–2 weeks of drawing serum. ELISA was performed on same serum in November–December 1980.

[b] Adult, age unknown.

[c] Plague confirmed by isolation of *Y. pseudotuberculosis* subsp. *pestis*.

Table 2. Serological tests of human sera collected in Ovamboland during October–November 1980

| Patient | PHA titre[a] | ELISA titre[a] | | |
|---|---|---|---|---|
| | | IgG + IgM + IgA | IgG | IgM |
| 7-year-old girl, in hospital | < 1:4 | 1:256 | 1:64 | 1:32 |
| 3-year-old boy, in hospital | < 1:4 | 1:32 | 1:32 | 1:4 |
| 45-year-old woman, in hospital with large femoral bubo | < 1:4 | 1:512 | 1:256 | 1:64 |
| 8-year-old boy, in hospital with axillary bubo | < 1:4 | 1:256 | 1:64 | < 1:4 |
| 14-year-old girl, ambulatory out-patient | < 1:4 | 1:32 | < 1:4 | 1:32 |
| Seven other patients | < 1:4 | < 1:4 | < 1:4 | < 1:4 |

[a] Antibody to the specific F1 antigen of *Y. pseudotuberculosis* subsp. *pestis*.

(± 2 dilutions), possibly owing to a light "background" colouration occasionally present at these low serum dilutions. Non-specific ELISA reactions were not observed. Two sera that gave non-specific PHA reactions were negative by ELISA. Serodiagnosis for plague was identical by PHA and ELISA for 28 (93%) of 30 patients (Table 1). In two patients (7%) who were negative by PHA, ELISA detected significantly higher titres of F1 antibody in the convalescent sera, suggesting that positive diagnoses of plague were appropriate.

In contrast with earlier tests, the results of PHA done in 1980 differed from those of ELISA in 42% of patients. Five of 12 patients bled during October–November 1980 had F1 antibody titres of 1:32 or more by ELISA but all patients were negative by PHA (Table 2).

## DISCUSSION

Human plague in Ovamboland, Namibia, during the period 1976–80 was confirmed by isolations of *Yersinia pseudotuberculosis* subsp. *pestis* from patients. There were 2 bacteriologically confirmed cases in 1976, 3 in 1977, 18 in 1978, 3 in 1979, and 1 in 1980. Additional cases of plague were diagnosed on the basis of serological reactions, including those listed in this paper.

The results of PHA done during 1976–78 correlated better with the ELISA results than did those of PHA done in 1980. Our studies illustrate that differences in PHA reagents can occur even after every effort has been made to achieve standardization. Standardization may be easier to achieve with ELISA, especially if photometrically defined endpoints are employed that precisely measure differences between ELISA and corresponding EI tests.

ELISA was more sensitive than PHA for detecting plague antibody and increased the frequency of serodiagnosis of plague by 7–42% in this study. For screening sera, ELISA with a conjugate of pooled immunoglobulins (IgG + IgM + IgA) was used and can be recommended. Subsequent tests for IgG and IgM antibodies were informative. An ambulatory patient, who exhibited a clinical course characteristic of the "mild" plague frequently seen in Ovamboland (*3*, *4*), had an IgM titre of 1:32 but no IgG antibody. Patients with IgM antibody to F1 but without detectable IgG antibody have been detected in other studies employing radioimmunoassay (*9*). Additional case reports with appropriate clinical and laboratory data will be required to determine what interpretations are valid for this and other serological responses. Immunoglobulin patterns may indicate whether there are anamnestic responses in patients with pyrexial illnesses other than plague, or whether certain serological responses are representative of infections with atypical *Y. pseudotuberculosis* subsp. *pestis* (*10*).

ELISA can be recommended because of its sensitivity and versatility for measuring various types of immunoglobulins. However, the procedure for ELISA is somewhat more complicated than PHA and requires about 4 hours to complete when previously sensitized plates are used. Results are obtained in about 2 hours with PHA. The specificity of both tests can be ensured using inhibition controls; a reaction is non-specific if endpoints are not lowered when purified F1 antigen is added to the serum diluent.

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## RÉSUMÉ

### COMPARAISON ENTRE L'HÉMAGGLUTINATION PASSIVE ET LE TITRAGE AVEC IMMUNOADSORBANT LIÉ À UNE ENZYME POUR LE SÉRODIAGNOSTIC DE LA PESTE

Une étude a été effectuée pour comparer les avantages et les inconvénients du titrage avec immunoadsorbant lié à une enzyme (ELISA) et de l'épreuve d'hémagglutination passive classique (HAP) pour le sérodiagnostic de la peste humaine. Des sérums humains provenant du foyer pesteux d'Ovamboland en Namibie ont été examinés et on y a recherché les anticorps à l'égard de l'antigène de la fraction 1 (F1) de *Yersinia pestis*. Des paires de sérums recueillies en 1976–1978 à partir de 30 malades ont été éprouvées par HAP dans les semaines qui ont suivi le prélèvement, puis conservées dans des congélateurs jusqu'en novembre–décembre 1980 et examinées alors à l'aide de la technique ELISA. Douze autres sérums non appariés, recueillis en octobre–novembre 1980 de cas pour lesquels un diagnostic présomptif de peste avait été posé sur la base des symptômes cliniques, ont été éprouvés par les deux méthodes sérologiques. Une augmentation de quatre fois ou plus du titre jusqu'à 1:32 ou plus, ou bien un titre de 1:32 ou plus accompagné d'une symptomatologie typique et sans antécédents de vaccination antipesteuse ont été considérés comme des critères suffisants pour un diagnostic positif de peste.

La technique HAP a été effectuée selon la méthode recommandée par l'OMS et on a retenu comme titre la dilution de sérum la plus poussée donnant une hémagglutination complète. Dans le cas de la technique ELISA (qui est décrite), le titre a été la dilution de sérum la plus poussée produisant une réaction colorée nette. La spécificité de la technique ELISA a été déterminée par l'exécution simultanée d'épreuves d'inhibition d'ELISA (IE), qui ne différaient d'ELISA que par le fait que le diluant utilisé pour préparer les dilutions de sérum contenait 100 $\mu$g de F1/ml. Le titre ELISA était considéré comme une mesure de l'anticorps spécifique de F1, si IE avait un point final au moins huit fois inférieur à celui d'ELISA.

Les résultats obtenus par la technique HAP en 1976–78 étaient les mêmes que ceux qui ont été obtenus avec ELISA. Les sérodiagnostics de peste étaient identiques pour 28 des 30 malades, soit 93%. Deux malades (7%) étaient négatifs en HAP, alors que ELISA a révélé des titres nettement plus élevés d'anticorps anti-F1 dans les sérums de convalescents, suggérant qu'un diagnostic positif de peste était justifié. Les titres obtenus par les deux techniques étaient similaires et pour l'une et l'autre l'erreur était normalement de $\pm 1$ dilution d'ordre 2; cependant, les résultats fournis par ELISA pour les sérums dont les titres étaient compris entre 1:4 et 1:16 étaient un peu plus variables ($\pm 2$ dilutions).

La technique HAP appliquée en 1980 a fourni des résultats différents de ceux d'ELISA pour 42% des malades. Sur les 12 malades chez qui l'on a prélevé du sang en octobre–novembre 1980, 5 avaient des titres d'anticorps anti-F1 de 1:32 ou plus en ELISA, alors que tous ces malades étaient négatifs en HAP.

Nos études montrent qu'il se produit des différences dans les réactifs HAP, même lorsqu'on s'est efforcé le plus possible de les standardiser. La standardisation peut être plus facile à obtenir avec la technique ELISA, surtout si l'on utilise des points finals définis par photométrie, qui permettent de mesurer précisément les différences entre les épreuves ELISA et les épreuves IE correspondantes. La technique ELISA peut également être recommandée en raison de sa sensibilité et de sa capacité de mesurer différents types d'immunoglobulines (IgG, IgM, etc.). Le mode opératoire d'ELISA est cependant un peu plus compliqué que celui de HAP et exige environ 4 heures lorsqu'on utilise des plaques présensibilisées, alors qu'il est possible d'obtenir des résultats en 2 heures environ avec HAP.

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