

دراسة تمهيدية عن تطبيق استخدام بعض الاختبارات الحديثة
في الكشف عن أنتيجينات فيروس الطاعون البقري وأجسامه المناعية

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- تم في هذا البحث تطبيق بعض الاختبارات السيرولوجية الحديثة وتقييم استخدامها
في الكشف عن أنتيجينات فيروس مرض الطاعون البقري وأجسامه المناعية .
- فعند استخدام اختبار الترسيب في الاجار تمت محاولات لاستخلاص أنتيجين ترسيبي فعال
لفيروس مرض الطاعون البقري من خلال الزرع النسيجي باستخدام مادة NP-40 وأثبتت كفاءة
عالية .
 - كما استخدم اختبار تلامزة بروتين (أ) لميكروب العنقود الذهبي المقترن بالاجسام المناعية
في الكشف عن انتيجينات فيروس مرض الطاعون على الشريحة الزجاجية أو المعيارية
بالميكروتكنيك ووجد أن هذا الإختبار سهل الاستخدام سريع القراءة وأكثر حساسية من
اختبار الترسيب في الاجار .
 - كذلك أثبت اختبار الاليزا النقطية أنه أكثر الاختبارات حساسية في الكشف عن انتيجين
الطاعون البقري في خلايا الزرع النسيجي .
 - وبمقارنة تقييم عيارية السيرم المناعي لفيروس الطاعون البقري المحضر في الارانب
باستخدام كل من اختبار التعادل واختبار الاليزا الخلوي أثبت الأخير حساسية في ذلك
وسهولة تطبيقية في قياس عيارية الاجسام المضادة .
 - بالإضافة لما سبق هناك تقدم ملحوظ في الكشف عن انتيجين فيروس مرض الطاعون الموجود
في الاعضاء الداخلية للغدد الليمفاوية للحيوانات المصابه طبيعيا باستخدام اختبارات
تلازن بروتين الميكروب العنقود الذهبي والاليزا النقطية واختبار الترسيب في الاجار .

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**PRELIMINARY STUDIES ON THE APPLICATION OF SOME RECENT
TECHNIQUES IN THE DETECTION OF RINDERPEST VIRAL
ANTIGENS AND ANTIBODIES**
(With 3 Tables and Two Figures)

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SUMMARY

In this work, some of the recently applied serological techniques, have been developed and evaluated for the detection of rinderpest viral antigens and antibodies. Different methods have been adopted for the preparation of agar gel precipitating antigen. It has been proved that the NP-40 cell-extracted viral antigen is potent more reliable for detection of rinderpest viral antigens in tissue culture. SPA (staph. aureus Protein-A)-agglutination test was used as a slide and microplate assay and found to be easier, rapid and more sensitive than the Agar Gel Precipitation test (AGPT). Parallel to the above two techniques, the Dot-Immunobinding Assay (Dot-ELISA) has proved to be the most sensitive and versatile technique in detecting rinderpest viral antigen in tissue culture. In combination with the above mentioned methods, the titers of antibodies found in rabbit-anti-Rinderpest serum were estimated using the serum neutralization test and the cell-ELISA. It could be concluded that the cell-ELISA is a very sensitive reliable test which could be easily performed for the determination of antibody titers. A notable progress has been achieved for the detection of rinderpest viral antigens in organs and lymph nodes of naturally infected animals using the SPA-agglutination and Dot-ELISA test besides the AGPT.

INTRODUCTION

Notable advances in the development of laboratory technology for viral diagnosis have occurred in the last few years. Many of the recently developed rapid diagnostic techniques are in the category of tests for the detection of virus or viral antigen in tissues or other specimens independent of the cultivation of these viruses in the laboratory. In certain serious infections as in Rinderpest (RP), the rapidity of diagnosis is of paramount importance. The conventional methods used for serologic detection and quantitation of RP antigens or antibodies include serum neutralization (ROSSITER and JESSETT, 1982), complement fixation (NAKAMURA, 1958 and MOULTON and STONE, 1961), agar gel precipitation (WHITE, 1958; HUSSAIN and SARWAR, 1962; SCOTT, 1962 a,b, JOSHI *et al.*, 1972; SELVAKUMAR *et al.*, 1982). Fluorescent antibody technique (LIESS and PLOWRIGHT, 1963; LIESS, 1965). Immunoperoxidase (SELVAKUMAR *et al.*, 1981) and Enzyme-linked Immunosorbent Assay (ROSSITER *et al.*, 1981; ANDERSON *et al.*, 1982, 1983; SHARMA *et al.*, 1983).

The participation of some recent techniques for the identification and titration of sera in some viral diseases was the deriving force for the application of such methods in RP-infection. Cell-ELISA is a technique which has been utilized for the detection of viral antigens

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and antibodies using infected and fixed cell as a solid phase in microtiter plates (SAUNDERS, 1977; EL-SANOUSI, 1985). As a diagnostic tool, the Dot-ImmunoBinding Assay has been also used in some bacterial and viral infections (J. GORDON *et al.*, 1983). In addition to these techniques, the Staphylococcus aureus Protein-A agglutination test has been successfully used in the identification of some viruses like influenza (ZALAN and WILSON, 1976 and 1978).

The purpose of the present work is an attempt for standardization of such techniques for application in the diagnosis of rinderpest.

MATERIAL and METHODS

Virus and cells :

Rinderpest virus "Kabete O strain" was kindly obtained from the department of cattle plague (Veterinary Serum and Vaccine Research Institute, Rinderpest Unit, Abbassia). The virus has been propagated in BK cells for 102 passages, thereafter, three further passages on Vero cells have been done at Virology Lab. (Institute of animal Health, Dokki). In the Virology Lab (department of microbiology, Faculty of Veterinary Medicine, Giza), the virus was also propagated for one further passage on Vero cells.

Media and sera :

M199 and Eagle's Minimum Essential Medium (MEM), were purchased from "GIBCO" and used for the cultivation of Vero and BK cells respectively. Brain heart infusion "DIFCO" and nutrient broth containing tris-HCl 0.05 M, yeast extract 0.5% and lactalbumin hydrolysate 0.5% were used for the preparation of Staphylococcus aureus "Cown I strain", protein-A (SPA) suspension. Rabbit anti-RP virus hyperimmune serum was kindly supplied by RP-unit, ASF/BT division, A.V.R.I., Pirbright, Surrey, England. Fluorescein conjugated goat antirabbit was purchased from Behring AG. "Marburg, West Germany" and peroxidase conjugated goat anti-rabbit serum was secured from Miles. Negative rabbit preimmune serum was obtained from 2 months old normal rabbit.

Virus titration :

Titration of RP virus was carried out according to published procedures (ROSSITER and HESSETT, 1982), in microtiter plates. Infectivity titers were calculated according to REED and MUENCH (1938). 100 tissue culture infectious dose 50% (TCID₅₀), were used for serum neutralization, fluorescent antibody and cell-ELISA tests.

Preparation of different agar gel precipitating (AGP) antigens to RP virus:

Three types of AGP antigens were prepared from Vero cells infected with RP virus:

1. Freeze-thawed cell-extracted viral antigen prepared after the method adopted by RP unit, ASF/BT division, A.V.R.I., Pirbright, Surrey (1981).
2. NP-40 cell-extracted antigens, was prepared according to the described procedures adopted in type number 1, with additional modification made in our lab., where the sedimented cells, infected with RP virus, were resuspended in PBS (1/250 the original volume) to which NP-40 (non-ionic detergent) was added to a concentration of 0.5%. The detergent was left to act on ice at 0°C for 30 min. with periodical shaking, thereafter the cell-extract was centrifuged at 3000 revolution per minute (rpm) for 10 min. and then the supernatant was saved for use.
3. Supernatant tissue culture fluid of RP-infected Vero cells: After clear cytopathic changes have appeared, the tissue culture supernatant has been saved and clarified from cell debris by centrifugation at 3000 rpm for 10 min.

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A patent freeze-thawed cell-extracted antigen was kindly supplied from Pirbright and used as standard positive antigen.

Serological techniques

A. For detection of RP viral antigen: The following serological techniques were used for determination of RP viral antigen in infected tissue culture cells:

1. Agar gel precipitation test (AGPT), was done according to WHITE (1958), using undiluted rabbit hyperimmune serum against RP virus. This test was properly adopted for measuring the potency and validity of AGP antigens prepared above. The different antigens were left to react with the positive rabbit anti-RP serum under several environmental and preparative conditions.
2. SPA-slide agglutination test was performed according to described procedures after ZALAN and ILSON (1976 and 1978), as modified by EL-SANOUSI (1985). The SPA suspension was prepared according to the method adopted by KESSLER (1975). Rabbit anti-RP hyperimmune serum was originally incubated with the SPA-suspension to an end-dilution 1 : 10 before used in the slide test. Microplate-SPA agglutination test has been developed in our lab., after modification of the method described by ZALAN and WILSON (1976), in which the serum has been diluted 2-folds in phosphate buffered saline (PBS) and equal volumes of SPA-suspension (2%) were added. Serum-SPA mixtures were incubated at 37°C for 1 hour with periodical shaking at 5 min. intervals. Equal volumes of different viral antigens prepared above were then added. Plates were incubated at 4°C overnight and then the reaction was read.
3. Dot-Immunobinding Assay (Dot-ELISA): the test was done according to standard procedures described by HAWKES *et al.* (1982). 3 to 5 ul of the different prepared viral antigens (serially 10-folds diluted) have been dotted on nitrocellulose membrane and incubated with rabbit anti-RP (diluted 1 : 25), at 37°C for 2 hours. After washing, the membrane has been incubated with goat anti-rabbit peroxidase (diluted 1 : 500). The reading of the reaction was done after the addition of peroxidase substrate. All solutions and buffers used in the test are as mentioned by HAWKES *et al.* (1982), except that we have used 1% bovine serum albumin (BSA) and 0.5% Tween 80 in this buffered saline (PBS) as a blocking solution.

B. For detection of RP antibodies: The same serological techniques have been also used for estimation of antibody titers in rabbit (anti-RP hyperimmune serum). Serum neutralization test (SNT) was performed to act as standard comparative test. This SNT was done using the microtechnique adopted by ROSSITER and JESSETT (1982), in Vero cells.

Cell-ELISA: Vero cell monolayers in tissue culture microtiter plates, have been infected with 100 TCID₅₀ of RP virus and used as a solid phase for enzyme-linked immunosorbent assay (ELISA). Infected cells in the microplate were fixed 5 days postinfection with a fixation mixture composed of acetone 60%, ethanol 25% and methanol 15% for 5 min. at room temperature. The potency of viral antigens was estimated in the cell-ELISA system by using rabbit anti-RP hyperimmune serum (diluted 10-fold). Rabbit preimmune serum was used as a negative control. The test has been performed as adopted by EL-SANOUSI (1985).

RESULTS and DISCUSSION

A. Detection of RP viral antigens:

Performing a battery of AGPT, it has been proved that, best results were obtained using 1% agarose dissolved in distilled water and kept at 37°C. These optimal conditions have been used for the different viral antigens mentioned in table 1. For measuring the potency and validity of different RP-viral antigens prepared with different methods, it appears from the same table that the NP-40 cell-extracted antigen gave clear sharp line of precipitation after 72 hours.

On performing the SPA-slide agglutination test, 10% of SPA-suspension was incubated with rabbit anti-RP hyperimmune serum added to an end dilution 1 : 10. Equal volumes of the SPA-serum immunosorbent and viral antigens were mixed together on a microscope slide. The reaction was left to proceed and read within few seconds visually and microscopically.

The reaction of the SPA-serum immunosorbent with the freeze thawed antigen gave more heavier cell aggregates (Fig. 1 a) than that obtained with the NP-40 extracted antigen (Fig. 1 b). Negative reaction was given with the control rabbit preimmune serum (Fig. 1 c). Slight reaction with lower cell-aggregates has also prepared with the supernatant antigen as well as the reference Pirbright antigen as shown in table 1.

On the basis of the above mentioned reactions, it appears that the freeze-thawed concentrated viral antigen is a more reliable diagnostic material rather than the NP-40 antigen. This could be explained on the basis that the freeze-thawed cells produce and release more intact viral particles which were disrupted by NP-40 in the NP-40 cell-extracted preparation. The high solubility of the NP-40 extracted antigen may be less stable in the agglutination of antibodies-conjugated *Staph. aureus*.

On performing the dot-ELISA, the potency of the different prepared antigens was tested after they have been diluted 10 folds before being dotted on the membrane filter. According to the intensity of the reactions using rabbit anti-RP serum, (diluted, 1 : 25) and anti-rabbit peroxidase, end titers of the different antigens have been achieved. Table 1 presents also, these end titers and shows that, unlike the SPA-agglutination test, the NP-40 extracted antigen gave higher titers than those given by other antigen preparations. It seems also that the undiluted tissue culture supernatant could be used as a possible antigen preparation used as a dot antigen for the detection of RP antibodies. Figure 2 shows end titers of the different antigens as dotted reaction developing on the white background of the membrane filter. The expected higher titer of the NP-40 extracted antigen (table 1), is due to the action of the non-ionic detergent Non-Idet p-40 (NP-40), on the infected cells, resulting in complete destruction of the cell membrane and release of the cell associated virus particles and other viral soluble antigens.

Using the previous serological techniques, the different antigens have been used for the estimation of antibody titers in rabbit anti-RP hyperimmune serum. Table 2 presents the end titers given with the different antigens which were previously diluted 1 : 10 except the tissue culture supernatant used undiluted. from the table 2, it could be concluded that the AGPT is not so sensitive in comparison with the other tests like the SPA and dot-ELISA. It seems very clear that, the AGPT requires concentrated antigen and antibody preparations to give a convenient results and this is material consuming, besides that the test sometimes takes more than one day to show specific lines of precipitation. On the other hand, the dot-ELISA gave higher titers with the different antigen preparations rather than those which have been given with the SPA agglutination. It appears also from table 2, that the NP-40 extracted

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antigen is the most valid antigen preparation which could be used for the determination of antibody titers, due to the higher sensitivity of the test, which depends largely on enzyme reactions.

In combination with the previously mentioned binding techniques, it was the necessity to determine the titer of antibodies in the SNT and cell-ELISA. It seems very essential to assure that both tests depend on the use of living tissue culture cells which were inoculated with the virus-serum mixtures as in the SNT or fixed infected cells used as a solid phase as in the cell-ELISA. From table 3, it could be concluded that the cell-ELISA is a very sensitive reliable test which could be easily performed for the determination of antibody titers. In other work submitted for publication, a notable progress has been achieved for the detection of RP viral antigens in organs and lymph nodes of naturally infected animals using the SPA agglutination test and dot-ELISA besides the AGPT.

Determination of antibodies against RP virus in field serum-samples collected from vaccinated and naturally infected animals using the dot- and cell-ELISA, SPA and SNT is now in progress.

Table (1) Comparative detection and titration of rinderpest antigen by The AGPT, SPA and Dot-ELISA

Test	Source of antigen			
	T.C. fluid	Freeze-thawed	NP-40	Ref.
AGPT	—	+	+++	±
SPA	+	+++	++	+
Dot-ELISA	+	10	100	+
	(not diluted)			(no diluted)

AGPT : Agar Gel Precipitation Test.
 Dot-ELISA : Dot Immunobinding Assay.
 SPA : Staphylococcus Protein-A Agglutination.
 T.C. : Tissue Culture.

Table (2) Differential evaluation of rinderpest antigens in the AGPT, SPA and Dot-ELISA

Test	Type of antigen	Serum end titers
AGPT	1. Supernatant T.C. fluid.	< 2
	2. Freeze-thawed cell virus.	< 2
	3. NP-40 cell-extract.	< 2
	4. Reference freeze-thawed antigen.	< 2
SPA	1. Supernatant T.C. fluid.	64
	2. Freeze-thawed cell virus.	512
	3. NP-40 cell-extract.	64
	4. Reference freeze-thawed antigen.	64
Dot-ELISA	1. Supernatant T.C. fluid.	640
	2. Freeze-thawed cell virus.	1280
	3. NP-40 cell-extract.	2560
	4. Reference freeze-thawed antigen.	10

AGPT : Agar Gel Precipitation Test.
 Dot-ELISA : Dot Immunobinding Assay.
 SPA : Staphylococcus Protein-A Agglutination.
 T.C. : Tissue Culture.

Table (3) Comparative titers of rabbit anti-rinderpest serum in the serum neutralization test and cell-ELISA

Test	Serum End Titers
SNT	640
Cell-ELISA	10000

SNT : Serum eutraization Test.

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LEGENDS

- Fig. (1 a):** Shows heavy aggregates of SPA with rabbit anti-Rinderpestserum using the freeze-thawed cell virus.
- Fig. (1 b):** Presents the size and mode of dispersion of SPA aggregates with rabbit anti-rinderpest serum using the NP-40 extracted antigen.
- Fig. (1 c):** Shows fine sandy-like microscopical appearance of the SPA with rabbit-preimmune serum and rinderpest antigen.
- Fig. (2):** Shows end titers of rinderpest antigens as dotted reactions developing on the nitrocellulose membrane filter, using rabbit anti-Rinderpest serum.
1. NP-40 cell extracted antigen.
 2. Freeze-thawed cell antigen.
 3. Supernatant Tissue Culture fluid.
 4. Reference freeze-thawed antigen.

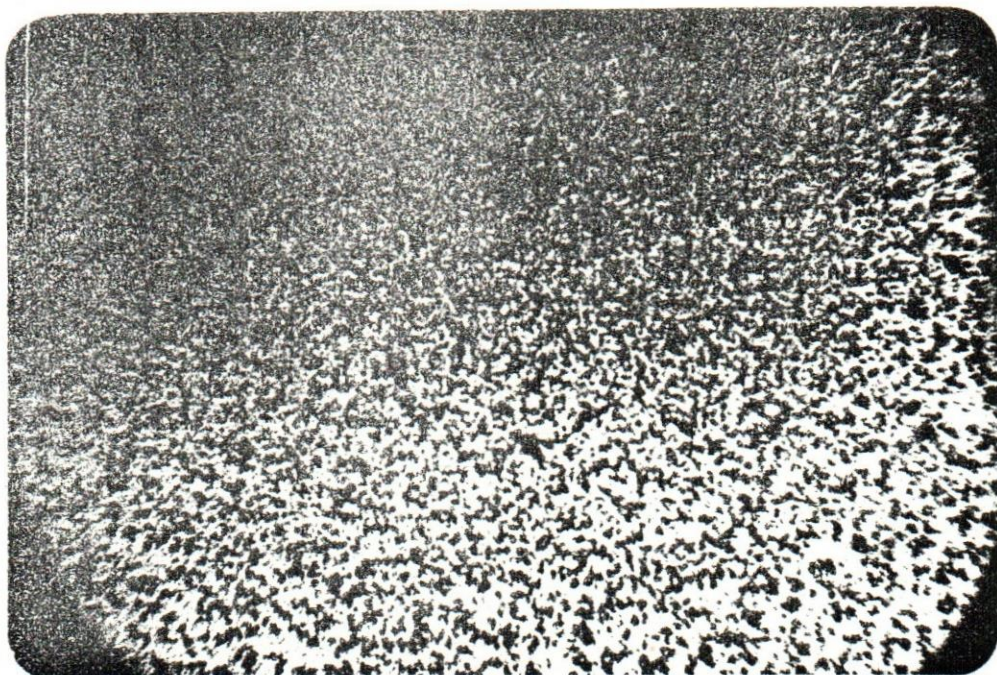


Fig. 1 a

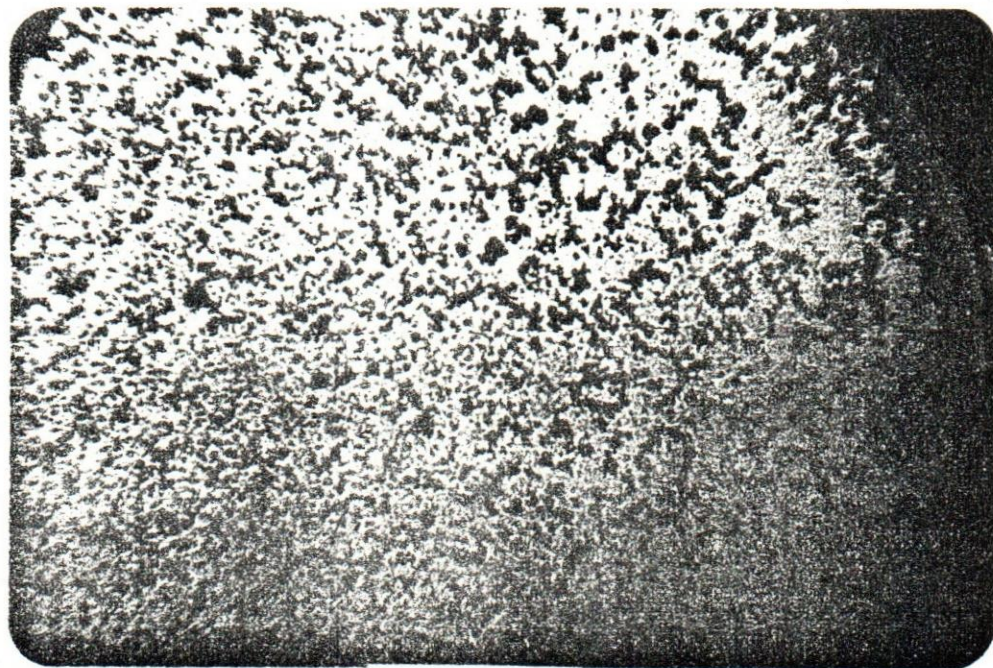


Fig. 1 b

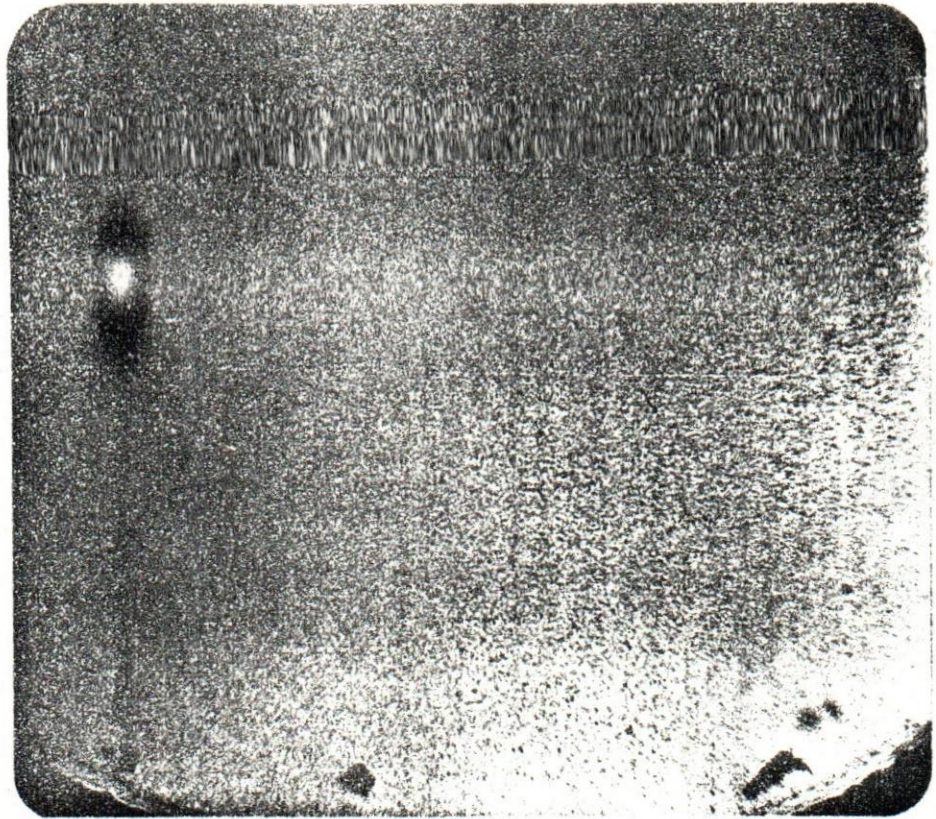


Fig. 1 c

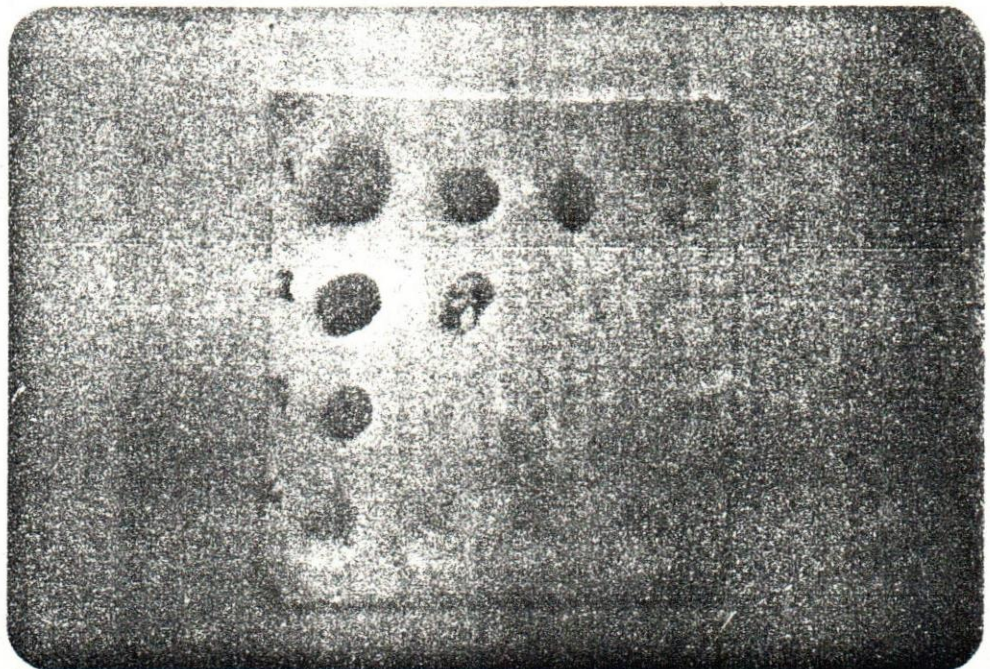


Fig. 2