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ARTICLE

A simple, effective and inexpensive method to highlight antigen-antibody reaction based on liquid-semisolid phase

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Abbreviations used: a2M, alpha-2-macroglobulin; Ab, antibody; Ag, antigen; Alb, albumin; DL, Detection limit RBP, retinol binding protein

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Abstract Identification of the immunological antigen-antibody reaction performed either in liquid phase or in agarose have several limits: antigen excess, low sensitivity, low speed reaction. A new method is described for the antigen-antibody reaction, easy, simple to perform and inexpensive. It is based on a reaction where the specific antibody is mixed with a semi-solid gel on a transparent surface and the antigen to be identified is inoculated into the gel. In this method, an immune precipitate can be shown in just few minutes, obtaining a good sensitivity compared with manual methods such as the Ouchterlony technique and other similar methods. By using albumin, IgG, RBP, transferrin, alpha-2-macroglobulin as antigen, the immune precipitate is formed within 10 min at 2–8 microgram/ml and, after 30 min, at 1–4 microgram/ml may be detected. The display of the immune precipitate is favored by oblique observation on a black background with a perpendicular light illuminating the gel from the bottom upwards. This method also allows a good control of antigen excess, a problem that is instead found in immunoturbidimetric reactions. The method here described has also been tested in serum and urine, and then it can be advantageously used as qualitative or semi-quantitative immunological test in research or diagnostic. Moreover, it could be particularly useful in low resources geographical areas, where an immunoassay that requires the use of dedicated equipment is not economically sustainable. The authors have waived the patent rights to allow the free development of new tests based on this method.

Keywords: agarose, antigen-antibody reaction, antigen excess, immunodiffusion, inexpensive method

INTRODUCTION

The state of the art of the antigen-antibody reactions (Ag-Ab) without the use of amplification systems (for example: enzymes) consists in the liquid-liquid reactions with manual methods with the aid of a spectrophotometer and liquid-liquid with automatic methods (turbidimetry or nephelometry) or manual methods, liquid-solid (reactions in agarose), such as the Ouchterlony technique. The limit of these methods in the liquid phase is given by the difficulty in controlling any excess of Ag or Ab with the possibility that the formation of the immune precipitate does not occur [1,2]. The problem can only be overcome by performing a sufficient number of dilutions of the Ag or the Ab in order to find the right balance. The manual operation is complicated and makes the analysis more costly as each dilution corresponds to a further test to be performed. Alternatively, to overcome these problems, it is necessary to have sophisticated automatic equipment such as a nephelometer or a turbidimeter, which solve the problem by evaluating the intensity of the reaction at different times and determine the presence of any Ag excess [3-5] according to the profile of the reaction. To overcome the problems of liquid-liquid reactions without resorting to expensive equipment, immunological identification techniques in agarose have been developed [6,7]. These exploit the peculiarities of the solid phase and allow the reagent and the analyte [6] or only the analyte [7] to diffuse through the mesh of the agarose itself and to form a concentration gradient reaching the optimum concentration of Ag-Ab in a point in the area of the plate. These techniques are penalized in practice by the long diffusion time-scales required. The reading timing of the reaction may take from several hours to several days. A further limit is given by the dilution of the reagent and of the analyte that necessarily occurs during the diffusion of the agarose, with a consequent reduction of the analytical sensitivity. On the basis of these considerations, a method has been developed which uses a liquid phase to a semi-solid phase [8,9][1]. The liquid phase is represented by the sample to be analyzed and the semi-solid phase consists of a gel in which the specific Ab is mixed. The semi-solid gel is distributed onto an appropriate reaction area and the sample is dispensed (inoculated) inside the gel. A liquid phase is

1 The authors have decided to let a patent granted by the Italian State expire [8] and didn't complete the international patent application [9] in order to allow anyone to further develop and create analytical applications based on this technique without the need for payment of economic rights.

more similar to a semi-solid phase than a solid phase, therefore, the contact and interpenetration between a liquid phase and a semi-solid one is favored compared with that between a liquid phase and a solid one. As a result, a liquid-semi-solid reaction allows obtaining a speedier immune precipitation. Since the reaction takes place in a short time and in direct contact between Ag and Ab, there is less dispersion of the reagents and a higher sensitivity will be obtained compared with the traditional methods. This technique takes advantage of the greater speed of penetration and contact between the two phases whilst maintaining a sufficient degree of separation between them and thus represents an advantage in the control of the Ag excess compared with the liquid-liquid reaction. The purpose of this study is to: 1) demonstrate that the greater dynamism of the system allows carrying out the reactions that occur in the solid-phase techniques in much narrower times and with less diffusion of the reagents and to solve problems that arise in liquid phase techniques; 2) offer an easily feasible, effective and low cost method to highlight the Ag-Ab reaction, usable both in the field of research, for example for the possibility of using small reaction volumes, and in those geographic areas which do not have sophisticated equipment, but which have the need to identify some analytes of diagnostic interest.

MATERIALS AND METHODS

Choice of the semi-solid medium

Methylcellulose (Sigma-Aldrich Corporation,St. Louis, MO, USA) is used as an inert medium in pharmacological testing because it is non-toxic and presents low interactions with most molecules. An acceptable balance in the viscosity of the gel must allow the visually evident formation of the immune precipitate, favoured by a viscous gel, and a smooth dispensation of the gel, favoured by a fluid gel. The optimum viscosity for this method is achieved when, taking into account the above conditions, the separation of the liquid phase (sample) and the semi-solid phase (semi-solid gel) is maintained after the dispensation of the sample. The required characteristics were obtained as follows: 1) a circular shape reaction area was taken into consideration because it is the natural expansion of a liquid droplet, and 2) the diameter of the reaction area must be sufficiently large to allow a proper viewing of the reaction and at the same time not too large in order to allow a homogeneous dispensation of the gel. Therefore, an area was chosen consisting of a virtual circle of 1 cm in diameter. The tests were performed by dispensing 50 µl of methylcellulose onto an appropriate transparent gel holder. Different densities of methylcellulose were used as stated by the manufacturer: 15 centipoises (cp), 25 cp, 400 cp, 1500 cp, 4000 cp at a temperature of 20°C for an aqueous preparation at 2% by weight/volume.

Reaction gel holder

The immune precipitation reactions were conducted in open and closed Petri dishes, and on glass gel holder.

Preparation of the methylcellulose

A viscosity allowing a good dispensation on the glass by simply using a micropipette was identified. The gel should not be too thick otherwise it remains retained in excessive amounts in the tip and it must not be too fluid otherwise it spreads on the glass beyond the provided area. To seek the optimal viscosity, every methylcellulose was prepared with the following concentrations: 1.6%, 2.0%, 2.4%. A quantity of 50 µl was taken from each mixture and the actual amount discharged from the tip was then weighed. The weight obtained was then subtracted from the theoretical weight of the withdrawn amount, obtaining in this way the amount of gel retained by the tip at a specific concentration. The higher concentration was chosen, i.e. the higher viscosity, such that the loss of material was not greater than the arbitrary value of 10%. The optimal density was then obtained by mixing four parts of methylcellulose 400 cp at 2% and one part of antiserum, thus obtaining a final concentration of methylcellulose of 1.6%.

Quantity of sample to be used

Bromophenol blue was employed (Sigma-Aldrich Corporation) at a concentration of 0.2 g/L in phosphate buffered saline (PBS) mixed with bovine serum albumin (Alb)-fraction V (Sigma-Aldrich Corporation) at the concentration of 1% (weight/volume), in order to simulate the diffusion of a protein sample within the drop of methylcellulose and thus to assess in which area the liquid phase (sample) is positioned in the semi-solid phase (gel). Different amounts of colored sample were inoculated (5 µl, 10 µl, 20 µl) into the gel without mixing the two phases. The diffusion of the sample in the gel was then assessed and a quantity of the sample was chosen in order to still leave a free area allowing a comparison between the reaction zone and the area where there is no reaction. The optimal amount of sample to be dispensed was 5 µl (1/10 of the amount of gel used).

Immunological response of Ag-Ab

All reactions were performed at room temperature. By keeping the percentage of gel unchanged, a portion of the buffer was replaced by the same percentage of Ab (**Table 1**).

Table 1. Schema of the reagent mixtures (A, B, C) at different Ab concentrations.

Ag (Mix)	Gel %	Buffer A %	Antiserum %	Antiserum final concentration (mg/L)
Alb (A)	80	0	20	2800
Alb (B)	80	14	6	840
Alb (C)	80	18	2	280
IgG (A)	80	0	20	1640
IgG (B)	80	14	6	492
IgG (C)	80	18	2	164

With these preparations the tests of reactivity were performed by using anti-Alb (polyclonal rabbit anti-human albumin, titre 2800 mg/L [10], Dako Cytomation, Glostrup, Denmark) and anti-IgG antibodies (polyclonal rabbit anti-human IgG, titre 3800 mg/L, Dako Cytomation) and the relative Ag: Alb (MW 66 KDa) and IgG (MW 150-170 KDa) (human serum protein calibrator, Dako Cytomation) at different concentrations. Results have been evaluated at different times.

Visual analysis of the immune precipitate

The formation of the immune precipitate was observed at a 30°-45° angle on a black background by using a light perpendicular to the gel

(**Fig. 1**) [11].

In **Figure 2** it can be observed a negative reaction and 5 immune precipitates at different concentrations of Alb using mixture "A", in which the immune precipitate was highlighted in the form of a ring or a circular area. The reaction time was 30 min for all conditions. The qualitative evaluation is highlighted in the figure legend.

The reactivity for 1000 mg/L and 10000 mg/L conditions (panels E, F) are similar indicating that a plateau phase has been probably reached.

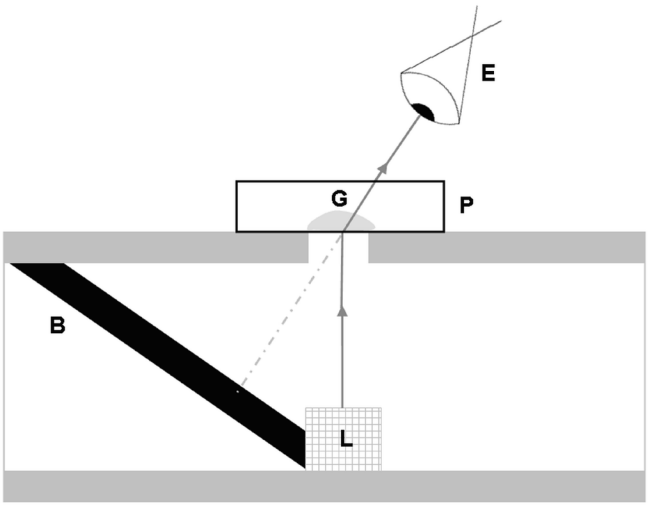

Figure 1. Model of the visualization system. Schematics showing the set up for visualizing formation of the immune precipitate. L = Lamp, B = Black background, P = Petri dish, G = Reaction gel, E = Eye.

Detection limit (DL) and speed of reaction

An evaluation of the DL and the speed of reaction of the method was performed by preparing, both for the Alb and for the IgG, three different concentrations of antiserum (**Table 1**). Each experimental condition was evaluated, according to the score previously defined, at times varying between 10 min and 40 min.

Reactivity with high quantities of Ag

With the mixtures "A" described in **Table 1**, a test of precipitation was performed with high amounts of Ag in order to demonstrate the ability of the method to control the effects of the Ag excess: for this purpose the calibrator Alb at a concentration of 100,000 mg/L and the IgG calibrator at 20,000 mg/L were used.

Reactivity with other proteins

Three more proteins with different molecular weights (MW) have been analyzed: retinol binding protein (RBP), MW 21KDa (N Protein Standard SL, Dade Behring, Germany); transferrin (Tf), MW 80 KDa (Human Serum Protein Calibrator, Dako Cytomation) intermediate between Alb and IgG; alpha-2-macroglobulin (a2M), MW 780 KDa (human serum protein calibrator, Dako Cytomation) higher than IgG. The antibodies used for the reactions were: polyclonal rabbit anti-human RBP, polyclonal rabbit anti-human tranferrin, polyclonal rabbit anti-human a2M (Dako Cytomation).

Analyses were performed using the optimal conditions obtained by Alb and IgG experiments: 50 µl reaction gel (**Table 2**), 5 µl of sample, closed Petri dish, 30 min reaction time.

Reactivity with serum and urine

Two human sera from healthy subjects and 4 urine (one from a normal subject and 3 from nephropathic patients) have been analyzed for RBP, Alb, tranferrin, IgG and a2M. Sera were diluted 1:10, 1:100, 1:1000 and 1:10000 with PBS, due to high protein concentration. Urine were used undiluted. Reaction conditions were: 50 µL reaction gel (Alb and IgG see **Table 1**, Mix A, RBP, Tf and a2M see **Table 2**), 5 µl of sample, closed Petri dish, 30 min reaction time.

As reference, the same sera and urine have been previously analyzed for the same antigens by a nephelometric method (Siemens BN2, Germany).

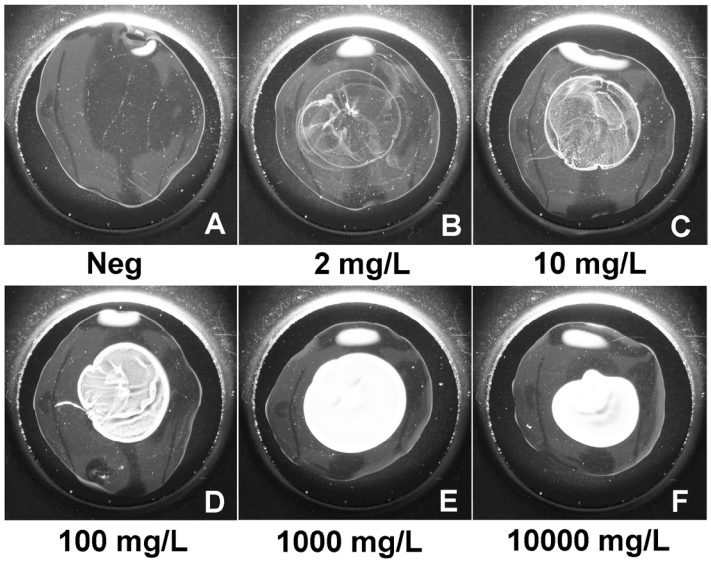

Figure 2. Images of the immune precipitates at different concentrations of IgG The reactions were carried out in closed Petri dishes using the indicated concentrations of IgG. Negative reaction (Neg) was included as a control. Images in panel A-F represent criteria used for assigning scores. The qualitative evaluation of the results obtained is expressed by using the following score: - (panel A), + (panel B), ++ (panel C), + + + (panel D), + + + + (panel E, F).

Table 2. Schema of the reagent mixtures.

Ag	Gel %	Buffer A %	Antiserum %	Antiserum final concentration (mg/L)
RBP	80	0	20	820
Tf	80	0	20	2200
a2M	80	0	20	880

RESULTS

Choice of the gel holder

In **Figure 3**, it can be observed how the gel presents after 30 min if it is deposited on an open Petri dish (A), on a closed Petri dish (B) and on a glass plate (C).

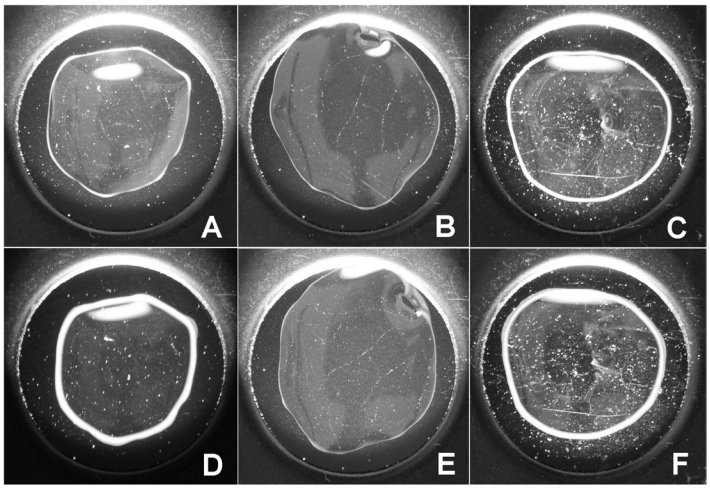

Figure 3. Images of gel appearance under different conditions. It can be observed how the gels appear after 30 min if deposited on an open Petri dish (A), on a closed Petri dish (B) or on a glass dish (C). Panels D, E, F show the same gels as they appear after 60 min.

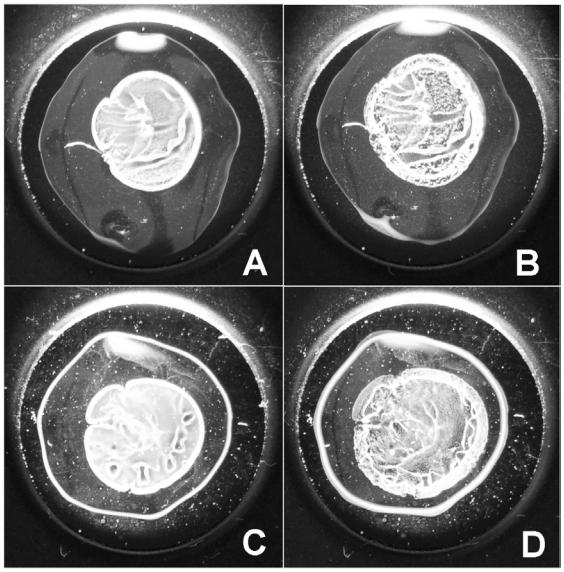

Figure 4. Gel images of Ab-Ag reaction. Ab-Ag reaction with IgG at a concentration of 100 mg/L both in a closed Petri dish in A and B and in an open Petri dish in C and D**.** The images A and C show the immune precipitate after 30 min and images B and D after 60 min.

Table 3. Evaluation of the sensitivity of reagent mixtures A, B and C (described in Table 1). The results are shown, for each mixture, at different time points. The visual evaluation was performed according to the criterion described in **Figure 2**.

Ag (mix) - time	64 mg/L	32 mg/L	16 mg/L	8 mg/L	4 mg/L	2 mg/L	1 mg/L	0.5 mg/L
Alb (A) – 10’	+++	+++	+++	+++	++	+	±	-
Alb (B) – 10’	+++	+++	+++	+++	++	+	-	-
Alb (C) – 10’	+++	+++	+++	+++	++	+	-	-
IgG (A) – 10’	+++	+++	+++	++	+	±	-	-
IgG (B) – 10’	+++	+++	+++	++	±	-	-	-
IgG (C) – 10’	+++	+++	+++	++	±	-	-	-
Alb (A) – 20’	+++	+++	+++	+++	++	+	+	±
Alb (B) – 20’	+++	+++	+++	+++	++	+	±	-
Alb (C) – 20’	+++	+++	+++	+++	++	+	-	-
IgG (A) – 20’	+++	+++	+++	++	+	±	-	-
IgG (B) – 20’	+++	+++	+++	++	±	±	-	-
IgG (C) – 20’	+++	+++	+++	++	±	-	-	-
Alb (A) – 30’	+++	+++	+++	+++	++	+	+	±
Alb (B) – 30’	+++	+++	+++	+++	++	+	+	-
Alb (C) – 30’	+++	+++	+++	+++	++	+	-	-
IgG (A) – 30’	+++	+++	+++	++	+	+	-	-
IgG (B) – 30’	+++	+++	+++	++	+	±	-	-
IgG (C) – 30’	+++	+++	+++	++	±	-	-	-
Alb (A) – 40’	+++	+++	+++	+++	++	+	+	±
Alb (B) – 40’	+++	+++	+++	+++	++	+	+	-
Alb (C) – 40’’	+++	+++	+++	+++	++	+	-	-
IgG (A) – 40’	+++	+++	+++	++	+	±	-	-
IgG (B) – 40’	+++	+++	+++	++	+	±	-	-
IgG (C) – 40’	+++	+++	+++	++	±	-	-	-

In the case of the open Petri dish and the glass plate a white ring is highlighted on the edge of the gel caused by the dehydration of the gel. After 60 min, as shown in the three images below (panels D-F), the ring is even more pronounced. This ring, however, does not interfere with the reaction: in fact, in **Figure 4** it can be observed how the visualization of the precipitate of the IgG in both the closed Petri dish (in panels A and B) and in the open Petri dish (in panels C and D) does not undergo modifications by the outer ring.

The closed Petri dish proved to be the best gel holder as it can limit the dehydration of the sample and because it offers a greater protection to the reaction system, as shown in **Figure 3**, where the images on the left show the immune precipitate after 30 min and those on the right after 60 min.

DL and speed of reaction

The results of the DL method and the rate of the reaction are shown in **Table 3**.

Mixture "A" has proven to be the most effective in terms of sensitivity and speed of reaction: 10 min since the beginning of the reaction the Alb is highlighted at a concentration of 2 mg/L and the IgG at 4 mg/L; at 20 min and 30 min the minimum detectable concentration of Alb is reduced to 1 mg/L; at 20 min the IgG is evident at a concentration of 4 mg/L, and at 30 min at 2 mg/L.

Reactivity with high quantities of Ag

It has been demonstrated that a highly visible immune precipitate is formed also in the presence of high amounts of Ag. The test carried out with Alb at 100,000 mg/L and IgG at 20,000 mg/L already showed a marked reactivity at 10 min and the immune precipitate was stable up to 1 hour before beginning to show some signs of gel dehydration (**Fig. 5**).

DL and speed of reaction of RBP, Tf and a2M

The results of the DL method and the rate of the reaction are shown in **Table 4**.

Method application to a clinical setting

The method, whose optimal conditions have been previously defined, has been applied to detect five antigens in human sera and urine. These antigens have been also quantitated in the same samples by nephelometric assay as reference (**Table 5**).

Results obtained from serial dilutions of sera and undiluted urine samples are summarized in **Table 6**.

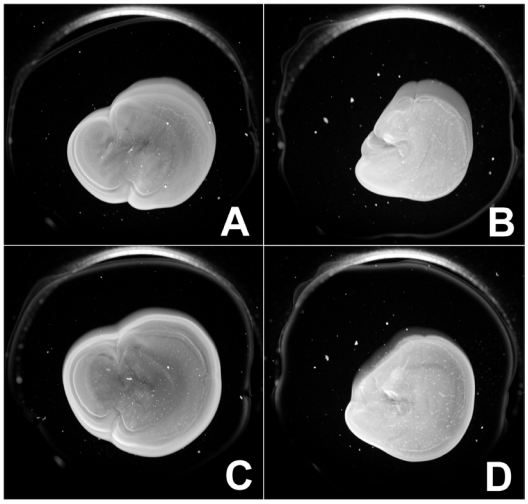

Figure 5. Ab-Ag reactions with Alb and with IgG at high concentration. Panel A shows the reaction with Alb at 100,000 mg/L after 10 min and panel C after 60 min; panel B shows the reaction with IgG at 20,000 mg/L after 10 min and panel D after 60 min.

Table 4. Evaluation of the sensitivity of RBP, Tf and a2M. The results are shown at different time points. The visual evaluation was performed according to the criterion described in Figure2.

Ag – time	64 mg/L	32 mg/L	16 mg/L	8 mg/L	4 mg/L	2 mg/L	1 mg/L	0.5 mg/L
RBP – 10'	+++	+++	+++	++	+	±	-	-
Tf –10'	+++	+++	+++	++	+	±	-	-
a2M – 10'	+++	+++	+++	++	±	-	-	-
RBP – 20'	+++	+++	+++	+++	++	+	±	-
Tf –20'	+++	+++	+++	+++	++	++	±	-
a2M – 20'	+++	+++	+++	++	+	±	-	-
RBP – 30'	+++	+++	+++	+++	++	+	±	-
Tf – 30'	+++	+++	+++	+++	+++	++	+	±
a2M – 30'	+++	+++	+++	++	+	±	-	-

DISCUSSION

In the present paper, a particularly simple and economical method to highlight an Ag-Ab reaction has been described. With simple equipment, such as a portable viewer, a pipette, a Petri dish or a glass plate and reactive gel, useful indications can be obtained concerning analytes that require a qualitative response. We first analyzed two antigens commonly found in serum, Alb and IgG, and for these antigens

the best protocol consisted of a 30 min reaction with the mixture A in a closed Petri dish. Using the same protocol we analyzed other antigens with various MW (RBP, transferrin, a2M), obtaining good sensitivity data. Moreover, the same five antigens have been evaluated in real clinical settings, such as serum and urine, proving the applicability of the described method to these biological fluids when using the correct dilution. This method can then be a valuable tool to assess the presence of Ag at concentration of few µg/ml: for that reason it can be employed both in research laboratories or in particular situations in which sample amount represents an issue. The tests carried out with the open or closed reaction gel holders have shown that the method is adaptable to various operational situations without undergoing significant changes. The reaction area was not limited by wells or barriers: this allows a greater versatility of the method in terms of the volumes of reaction. In this way the consumption of the reagents and the visualization of the reaction may be effectively adjusted. This method could be adapted to smaller reaction areas, with the aid of viewer-enlargers, in order to visualize the presence of analytes such as the monoclonal Ab in unknown supernatants. The use of a reactive gel, contained in a vial, makes the method "sturdier" than a plate of agarose [6,7] which is more susceptible to drying out and to transport damage. Our method can also be considered a feasible alternative when two different antibodies directed against the same antigen are not available and therefore the "sandwich" method cannot be applied. Another possible application could be the detection of specific antibodies against a known antigen mixed with the reaction gel. A practical example may be represented by the determination of the anti-typhoid and paratyphoid antibodies [12,13].

Table 5. Nephelometric assay on sera and urine. See Materials and Methods for samples description.

Ag	RBP mg/L	ALB mg/L	Tf mg/L	IgG mg/L	a2M mg/L
Serum A	46	42400	2600	12300	3100
Serum B	42	48300	2100	9400	3600
Urine 1	0	12	1	0	0
Urine 2	5	2827	180	359	66
Urine 3	0	431	37	95	18
Urine 4	36	207	9	72	0

This study shows that the method described can have its place in the landscape of immunological techniques. The philosophy of the method is, however, to offer a simple technology, versatile and inexpensive which may find its main application in research or in critical situations where, either for economic or logistical reasons, no other alternatives are available.

Table 6. Results from serial dilutions of sera and undiluted urine samples. The visual evaluation was performed according to the criterion described in **Figure 2**.

Ag – dilution (dil)	RBP	ALB	Tf	IgG	a2M
Serum A – 1:10	+	++++	+++	++++	+++
Serum A – 1:100	-	++++	+++	+++	+++
Serum A – 1:1000	-	+++	+	++	±
Serum A – 1:10000	-	++	-	±	-
Serum B – 1:10	++	++++	+++	++++	+++
Serum B – 1:100	-	++++	+++	+++	+++
Serum B – 1:1000	-	+++	++	++	+
Serum B – 1:10000	-	++	-	-	-
Urine 1 – NO dil	-	+++	±	-	-
Urine 2 – NO dil	++	++++	++++	++++	+++
Urine 3 – NO dil	-	++++	+++	+++	+++
Urine 4 – NO dil	+++	+++	+++	+++	-

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