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Chapter

Semi-Solid Phase Assay for the Alternative Complement Pathway Activity Assessment (AP_{100})

Kheir Eddine Kerboua and Kamal Djenouhat

Abstract

Since the introduction of the most expensive drug in the world (Eculizumab) in the therapeutic arsenal of many diseases involving the alternative complement pathway (ACP) in their pathophysiology, the unmet need to perform simple ACP assays affordable for all countries has become one of the major challenges of the contemporary medicine. The assay currently used is AH_{50}, despite it still challenging for several laboratories. This educational chapter consists on a detail protocol of standardized hemolytic assay AP_{100} and aims to help clinical laboratories over the world and especially those of the developing and low incomes countries to perform it. The procedure is essentially the same as for the timed lysis assay and dilution methods (AP_{50}) except the concentration of ACP buffer and the chicken erythrocyte density used to make the gels. In clinical field, AP_{100} has at least nine applications in disease diagnosis and follow-up. AP_{100} has many advantages over the AH_{50} as it is more reliable for the Eculizumab monitoring and more practical with a purpose to be stored and transported for several weeks. AP_{100} is a portable and easy to use device both at the bedside and in the companion medical care.

Keywords: alternative complement pathway, AP_{100}, medicine, simple assay, low income countries

1. Introduction

Complement system is the pillar of the immune system by its dual role in homeostasis and disease. It is the first line of the innate immunity and augments adaptive immunity. Indeed, complement acts as a rapid and efficient immune surveillance system that has distinct effects on healthy and altered host cells and foreign intruders through a complex cascade of proteases [1]. Activation of the pathway occurs through three primary pathways: classical, lectin, and alternative pathways. Instead to the other pathways and in addition to properdin as the initiating molecule, the alternative complement pathway (ACP) is activated via a low level of constitutive spontaneous hydrolysis of C3 in a process known as tick-over. Importantly, thanks to its amplification loop, ACP plays a major role for the final effect of initial specific activation of the classical and lectin complement pathways and contributed to 80–90% of any C5 activation regardless the initiating pathway [2]. Interestingly, ACP has been shown to play a particularly important role in preclinical disease models [3].

Disease presentation	ACP proteins involved
Immune complex (IC) diseases, SLE with and without glomerulonephritis (GN).	Most of ACP proteins trigger tissue injury because ACP constitutes the amplification loop of the classical pathway
Severe and often repeated pyogenic infections.	Deficiency of C3 either primary, or secondary to fH or fI deficiency, or the presence of C3 nephritic factors. Can also occur with classical component deficiency.
Mesangiocapillary glomerulonephritis (MCGN) or membranoproliferative glomerulonephritis (MPGN)	C3 deficiency classically caused by C3 nephritic factors, also fH deficiency
Neisserial infections, usually recurrent meningococcal infections. Occasionally disseminated *N. gonorrhea* infections.	Terminal component (C5–8) deficiencies; the association is less strong in patients with C9 deficiency. Patients are usually otherwise healthy.
Severe, occasionally repeated, *N. meningitidis* infections in males	Properdin deficiency or Factor D deficiency (very rare, not sex linked)
Paroxysmal nocturnal hemoglobinuria	Rare acquired clonal disorder of hemopoietic cells which lack glycosylphosphatidylinositol (GPI) membrane anchored Complement control proteins [(DAF) And CD59].

Table 1.
Clinical presentations of ACP proteins acquired or genetic abnormalities.

The ACP functional assessment constitutes an unmet need in medicine and applied research fields as the health valorization of bio-molecules extracted from nature. In clinical field it has at least nine applications in disease diagnosis and follow-up. For instance in therapy monitoring, it allows to screen patients responders to the complement blockers like Eculizumab, a patient with abolished activity means that he has no C5 mutation and is considered as eligible to this therapy. Furthermore, ACP activity makes possible to assess drug effectiveness at the plasmatic level [4]. Moreover, by evaluating ACP function we can predict and avoid immune-complex diseases flares and end organs damages as in systemic lupus erythematosus (SLE).

For the disease diagnosis, several international consensuses include functional hypocomplementemia and ACP abnormalities as a diagnostic criterion:

1. Kidney diseases resulting from abnormal control of ACP especially atypical hemolytic uremic syndrome (aHUS), C3 glomerulonephritis (C3GN), and dense-deposit disease (DDD), as well as atypical postinfectious glomerulonephritis [5].

2. Hypocomplementemia by a hemolytic assay constitutes one point in the diagnosis score of EULAR/ACR Lupus Classification Criteria 2017 and useful marker for evaluating SLE renal disease activity and outcomes [6].

3. The clinical hallmark of paroxysmal nocturnal hemoglobinuria (PNH) is the chronic intravascular hemolysis that is a consequence of unregulated activation of ACP [7].

4. Individuals deficient in components of the alternative and terminal complement pathways are highly predisposed to invasive, often recurrent meningococcal infections [8]. The most frequent bacterial meningitides related to complement proteins deficiencies are dues to factor B, factor D and membrane complex attack proteins deficiency.

5. Hypocomplementemic hypersensitivity reactions to synthetic hemodialysis membrane at the origin of cardiovascular complication, the most frequent and life-threatening complication in hemodialysis [9].

6. Several clinical presentations linked to ACP abnormalities as resumed in **Table 1** [2].

7. ACP is particularly considered in sepsis, due to its uncontrolled amplification in sepsis conditions [10].

2. Protocol

The procedure is essentially the same as for the ACP by kinetic fluid phase assay except the concentration of AP buffer and the chicken erythrocyte density used to make the gels [11]. A value of 100% of plasma ACP function should be defined by the pooled normal human plasmas (NHP standard), prepared from a total of 100 healthy individuals separate for each sex.

2.1 Samples

Patient serum samples for the functional hemolytic assays need to be fresh, that is serum should be separated on the day of venepuncture and used the same day, or stored at −80°C. This is probably the single most difficult, yet important, step because if the cold chain is broken, the results become impossible to interpret correctly [12]. Whole blood, with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant can also be used.

Chicken red blood cells (CRBC) was collected in tubes containing Alsever or 20% (v/v) acid citrate dextrose (ACD) and stored at 4°C.

2.2 Buffers and other reagents

1. Phosphate-buffered saline (PBS) contains: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO. Sodium azide can be added if required.

2. Alternative complement pathway buffer (ACP buffer) is PBS containing 100 mM ethylene glycol-bis(β-aminoethyl ether)N,N′,N′-tetraacetic acid (EGTA) and 7 mM $MgCl_2$; resulting in chelation of Ca^{2+}, but not Mg^{2+}, and providing additional Mg^{2+} [13]. This prevents complement activation via the classical pathway and facilitates complement activation via the ACP in agarose gels. ACP buffer/gelatin contains 1 g/L gelatin and is used when protein concentrations are low.

3. Agarose: use an agarose that has a low melting point as plates are easier to pour.

2.3 Procedure

1. Before starting, in a sterile tube put 9.8 mL of 1×ACP buffer at 56°C. Also let warming some Petri dish (or rectangular plate) in the 56°C incubator.

2. Preparation of chicken erythrocytes (CE) for ACP assay

a. Under aseptic conditions, remove 100 μl packed CE from stock chicken blood stored in Alsever's solution.

b. Wash twice in ACP buffer.

c. Resuspend in the same buffer to the required concentration for assay by 2.1 mL of ACP buffer and put the cell suspension in a water bath at a temperature of 46°C. The concentration of CE can be calculated by lysing 0.1 mL of the stock CE in 2.9 mL H_2O and measuring the absorbance of the supernatant at 412 nm.

3. Melt 2% agarose stock (most conveniently in a microwave oven although immersion in boiling water will suffice) and, using a warm pipet, pipet 12.25 mL aliquots into universal containers (one for each gel), keep at 56°C.

4. Add the 9.8 mL warmed 1×ACP (56°C) to each bottle of melted agarose. Mix well.

   Transfer one bottle with diluted agarose to 45°C and allow to cool to this tempe rature.

5. To ease pouring the gel, place the warmed Petri dish (or rectangular plate) on level tray. Mix carefully and quickly.

6. Pour the mixture evenly onto the level plate. The mixture should go to the mid edges of the Petri dish/rectangular plate. Remove bubbles by touching them with a pipette tip or gloved finger.

7. Cool plate to 4°C, punch holes using a Pasteur pipette upside down at least 1 cm apart.

8. Fill wells with a measured serum volumes (30 μL). Include a normal human plasma (NHP) standard, and NHP diluted 1/2 and 1/4 on each individual dish/plate. The size of rings depends on factors such as gel thickness, and NHP standards are needed on each dish.

9. Incubate overnight at 4°C, examine Petri dish before transferring to 37°C. Incubate at 37°C for 1–2 h. Incubate either overnight at 22°C and 2 h at 37°C.

10. Measure the diameters of the rings of lysis and calculate the areas. Areas of lysis can be read after photography, or after making direct photographic prints, but this is optional.

11. Standard curve: A crude standard curve is drawn by plotting % concentration NHP *vs.* area of lysis (diameter squared can also be used). This allows calculation of % normal activity in test samples.

3. Representative results

After incubation, the ACP activity is calculated after measuring the diameter using the vernier caliper or taking the ACP dish in photo and measure each surface well by *Image J*® (**Figure 1**). Area surfaces are calculated by the formula $S = \pi^* r^2$,

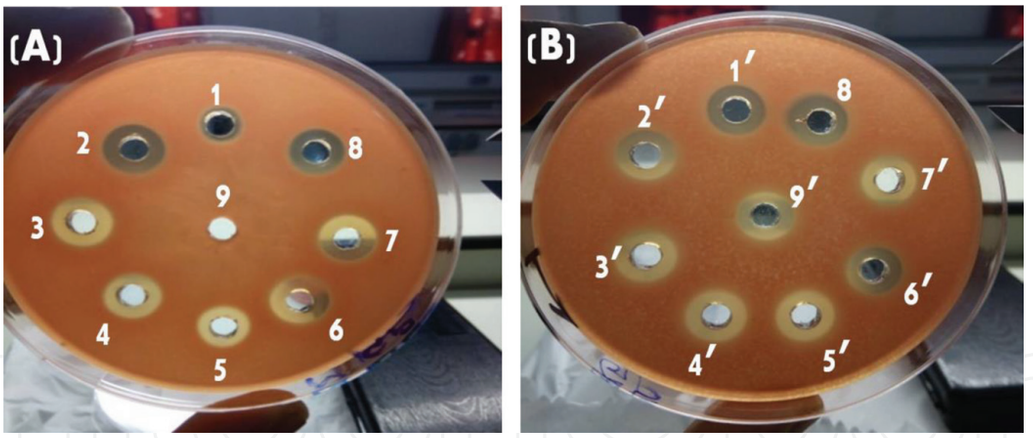


Figure 1.
ACP dishes for different sera from patients and pooled normal plasmas NHP. (A) Rings size of well 1 and 9 are found in a complete ACP blockade by Eculizumab or total deficiency of one of the ACP proteins. The remaining wells show different plasmatic levels of ACP. (B) Sera tested in this ACP dish are within normal range but vary according to the clinical presentation.

with r = d/2. (r: radius, d: diameter, s: surface). The same procedure is applied to the different NHP dilutions included to establish the calibration curve. This is because diluting NHP means that all ACP components are diluted equally. As example of interpretation, the more the ring is small the more the effectiveness of Eculizumab is good. The absence of reaction in a well signifies a complete ACP blockage, either by a drug or by a pathological process.

3.1 Materials

1. Chicken erythrocytes (CE) conserved in Alsever's solution.

2. Phosphate-buffered saline (PBS).

3. Veronal buffered saline (VBS).

4. Gelatin veronal buffer (GVB).

5. AP buffer is GVB containing 5 mM Mg and 5 mM ethylene glycol bis[β-aminoethylether]N,N′-tetraacetic acid (EGTA, E4378 Sigma).

6. N-saline is 9 g NaCl dissolved in 1 L H_2O.

7. Barbitone buffer is made by mixing 0.1 M solutions of sodium barbitone and barbituric acid to obtain the target pH and adjusting volume to obtain required final molarity.

3.2 Equipment

1. Suitable Petri dishes or glass plates. Size depends on the number of samples. Volumes can be adjusted so that the final depth of the gel is ~1–1.5 mm.

2. 56°C incubator and dishes/plate warmer (water bath can also be used).

3. 46°C water bath.

4. Level table.

5. 37°C incubator.

6. Pasteur pipette as well cutters to produce holes approximately 5 mm diameter. If single holes are to be punched, a grid should be placed under the gel so that the holes can be distributed evenly.

7. Refrigerated centrifuge.

8. Electronic balance.

9. pH meter/temperature probe.

4. Discussion

Since Thomas A. E. Platts-Mills and Kimishige Ishizaka have discovered that fresh normal human serum in EGTA buffer was found to cause >90% hemolysis of unsensitized rabbit red blood cells (RaRBC) [11], simple timed lysis assay and dilution methods called AP_{50} (Alternative Pathway 50) was performed to quantify hemolytic complement activity in human serum [13–15]. This reaction requires C3, factors B and D, and Mg^{++} ions to form the C3 convertase (C3bBb) [14, 15]. In AP_{50}, ACP is activated and measured by virtue of RaRBC decreased sialic acid content in addition to the blockade of the classical pathway activation by chelation of calcium by EGTA [16]. Nonetheless, AH_{50} requires a lot of material like spectrophotometer and consumable test tubes and highly skillful personal. To adapt this assay to simple labs, a semi-solid phase assay was proposed to measure ACP activity by using chicken erythrocytes incorporated in agarose gel called AP_{100}. Cell membranes of chicken erythrocytes have the same properties as those of the

	Fluid phase assay (AH_{50})	**Semi-solid phase assay (AP_{100})**
Number of samples	<5 per day	Several samples series per day
ACP Activators	Red cells	Red cells + agarose particles
End point assessment	Optical density by Spectrophotometer	Diameter measurement directly
Laboratory work flow steps	+++	Less
Time consuming	+++	Less
Storage	No	15–21 days
Transport	No	Yes (respecting the cold chain)
Global cost	+++	Less
Assay related error	+++	Less
Manipulator linked error	+++	Less
Others		• More suitable complement related diseases screening • Do not need a deep immunotechnology handling • Storage of utilized plates for *intra* and *inter*-laboratory comparisons

Table 2.
Hemolytic agarose assay features in comparison to tube hemolytic assay.

rabbit in terms of cell surface charge density of sialic acid whereas they are more robust than rabbit because they have nuclei. AP_{100} assay has many advantages over the AH_{50} one (**Table 2**). For example, AP_{100} is more reliable for the eculizumab monitoring as shown in the article we have recently reviewed [17]. Moreover, AH_{50} still challenging in low incomes country laboratories that cannot equip their hospital and research laboratories to perform this assay. To overcome this roadblock, it was proceeded to render this method more practical to each laboratory with a purpose to be stored and transported for several weeks. This includes a hemolytic agarose dishes/plates; a special adaptation to prevent complement activation via the classical pathway and facilitate complement activation via the alternative one in agarose gels.

To the best of our knowledge there were no laboratory performing that assays in developing countries especially in Africa and no educational chapter is available to explain it. Once its optimization in each of these countries laboratories was performed, thanks to ACP dishes/plates, we are expecting to empower doctors' decision making process and improve quality of patients' management and therapy follow-up. Therefore this portable and easy to use device even at the bedside of the patient and in the companion do not necessitate any equipped laboratory and may facilitate prospective analysis and disease screening in large populations. With enlargement of the ACP disease spectrum necessitated complement blockade, AP_{100} should be considered by clinical laboratory scientist especially to analyze sets of samples at once.

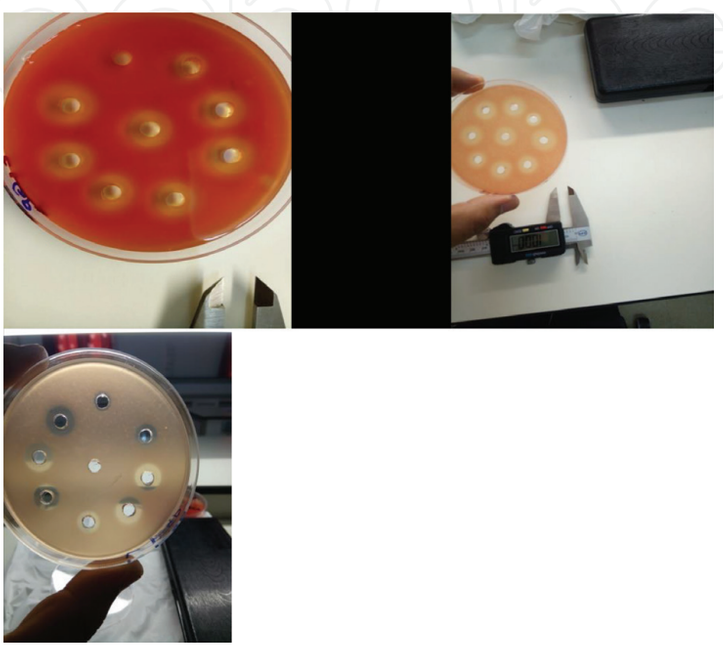
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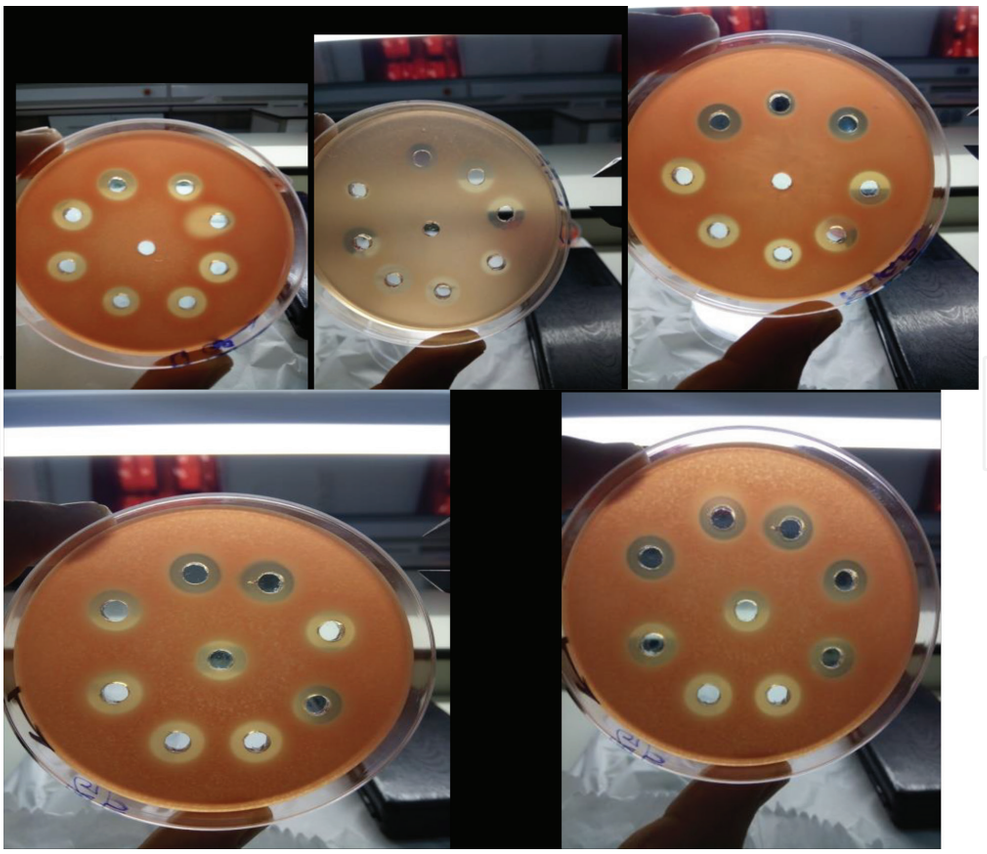
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Disclosures

The author has nothing to disclose.

A. Appendix





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