# Comparison of test tube, gel column agglutination and solid phase red cell adherence methods with and without drug added for detection of drug-dependent antibodies

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**ABSTRACT**

**BACKGROUND:** Testing for drug-dependent antibodies (DDA) is traditionally performed in test tubes either with drug-treated RBCs or in the presence of drug. Gel microcolumn and solid phase red cell adherence (SPRCA) methods were evaluated for detection of DDA with and without drug.

**STUDY DESIGN AND METHODS:** Forty-nine frozen DDA samples tested by tube methods were selected. Samples were tested with untreated RBCs without drug and in the presence of their identified drug by SPRCA and gel. RBCs treated with drug were tested by gel.

**RESULTS:** Gel showed similar reactivity to tube methods while SPRCA tests were weaker or negative. Thirty-five samples were positive by tube in the presence of drug, 34 by gel and 28 with SPRCA. With drug-treated cells, 22 samples were positive by both tube and gel; SPRCA was not tested. Without drug added, 9 were positive in tube, 13 in gel and 5 in SPRCA.

**CONCLUSION:** Gel was comparable to tube methods for detection of DDA by testing in the presence of drug and with drug-treated cells while SPRCA was less sensitive for detection of some DDA. Gel increased detection of antibodies without added drug.

## INTRODUCTION

Drug – Induced Immune Hemolytic Anemia (DIIHA) is a rare complication that has been estimated to occur in about one in 1 million individuals.[1] It is characterized by a sudden drop in hemoglobin in a patient following the administration of the putative drug. Some cases present with mild hemolysis while others can be quite severe or even fatal.[2] Serologic evaluation of these patients can be challenging. It is important to be able to distinguish if the hemolytic episode is caused by a drug and to confirm the presence of the drug-dependent antibody. Cessation of the offending drug will generally resolve the hemolytic episode.

Testing for drug-dependent antibodies has been based primarily on two methods.[1] Drugs may bind covalently to the red cell membrane and by treating normal RBCs with drug, the antibody can be demonstrated. The second type of drug-dependent antibody can be detected when the offending drug is present in soluble form in the test system either as the drug itself or a metabolite of the drug.

Most serologic investigations of DIIHA are based on test tube methods.[3,4]. Only a limited number of studies were found where gel technique was compared to tubes for the identification of DIIHA[5,6] and none were found where Solid Phase Red Cell Adherence (SPRCA) was used. Many institutions in the United States have moved to either gel or solid phase systems for the routine detection of antibodies in patient samples. This study compared gel and SPRCA against conventional tube techniques to determine if these methods are able to detect drug-dependent antibody in the presence of drug. In addition, an investigation of how drug-treated RBCs tested by gel would compare to tubes was performed.

This study also evaluated if there would be a change in the number of patient samples that would be reactive without the addition of drug to the test system using gel or SPRCA as compared to tube testing. A positive IAT on the initial serologic testing could give the appearance that hemolysis was caused by an auto- or alloantibody.[4,7]

## MATERIALS AND METHODS

Frozen, archived samples from patients with drug-dependent antibodies (DDA) previously confirmed by test tube methods were selected based on the volume of sample available for testing. Drug-dependent antibody testing was performed based on methods used in the original investigations. Fifteen different drugs were implicated. Antibiotics were the majority of drugs tested. Twenty-three samples involved cephalosporins and 13 samples were linked to penicillin and/or penicillin derivatives. There were seven samples where non-steroidal anti-inflammatory drugs (NSAIDs) were implicated and the remaining drugs, quinine, probenecid and oxaliplatin, had only a small number of samples available for this study. (Table 1)

Drug–dependant antibodies identified in the presence of drug (IPOD)

Powdered drug was dissolved in 6% albumin at a concentration of 1mg/mL. Patient samples were tested undiluted (neat) or with either the dissolved drugs or 6% albumin, as a dilution control, in equal parts.[8] Samples were evaluated using the testing systems listed below. Control samples were serum obtained from a normal donor known not to react with the drugs in question as well as a pool of six donor AB plasmas tested for non-reactivity with the indicated drugs.

- Tube Method

Two drops of sample (patient or control) plus either two drops of drug solution or 6% albumin were incubated with untreated RBCs for 30 minutes at room temperature, incubated for 60 minutes at 37°C and after four washes with saline, polyspecific Anti-Human Globulin (AHG) was added to the dry cell buttons to perform the Indirect Anti-globulin Test (IAT). Cell buttons were dislodged gently and results recorded. All samples negative at IAT were then confirmed by using IgG-coated RBCs. One of the patient samples required the use of diclofenac urine metabolite as the source of drug.[9] Urine was collected from an individual taking a therapeutic dose of diclofenac. The urine was centrifuged for 3-5 minutes and the supernatant removed. The pH was adjusted to 7.4. Samples were aliquoted and stored at -20°C until required for testing.

- Gel (MTS Anti-IgG Card™ , Ortho Diagnostics, Raritan, NJ) with Untreated Red Cells

Microcolumns containing gel and anti-IgG were utilized. Red cell suspensions of 0.8% from 2 different type O donors were made in MTS-Diluent 2™. Each sample (patient, control serum or pooled

AB plasma) was tested against both donor cells. In each respective gel microcolumn, 50 μL of cell suspension was added to the upper chamber and 25 μL of sample was added to the red cells. For drug studies, either 25 μL of appropriate drug suspension or albumin (control) was also added to the microcolumns. The columns were incubated for 30 minutes at 37°C then centrifuged at 893 rpm for 10 minutes. Reactions were graded according to manufacturer's directions.

- Solid Phase Red Cell Adherence (Capture-R® Ready-Screen® I and II, Immucor-Gamma, Norcross, GA)

Manufacturer's pre-made stripwells were utilized in testing. Each sample (patient, control serum or pooled AB plasma) was tested against both antibody detection wells unless the patient had an alloantibody matching the antigen profile in a particular well. Two drops of LISS were added to each test and control well. One drop of sample without drug was tested as well as one drop of sample and one drop of either drug solution or albumin (control) added to respective wells. Strips were incubated for 30 minutes at 37°C in a dry heat incubator. Wells were decanted and washed using an automated washer. After the final wash, 1 drop of indicator cells was added to each of the wells. Stripwells were centrifuged for 2 minutes at 530xg. Wells were then viewed for reactivity using a light box. Positive reactions displayed adherence to all or part of the reaction surface and were graded according to the manufacturer's instructions. Positive and negative test controls were provided by the manufacturer.

Drug Dependant Antibodies detected with Drug Treated Cells

O Negative (e-positive) red blood cells from donor pilot tubes were selected to serve as the negative control and for coating with drug. Powdered drugs were dissolved in barbital buffer (pH 9.6) at 300 mg/mL. The red blood cells were washed with normal saline and 0.5mL of the packed cells was mixed with 7.5 mL of drug solution. The cell-drug mixture was incubated for 60 minutes at room temperature with gentle agitation. The cells were then washed 4 times with normal saline. An aliquot of untreated washed cells was used as the normal control.[8]

- Tube Method

Patient samples were tested against the drug-treated red cells and untreated cells by incubating at room temperature for 15 minutes, at 37°C for 30 minutes and by IAT.

- Gel Method

A 50 µL aliquot of either drug-treated or untreated cells was added to the anti-IgG gel microcolumn and then 25 µL of sample was added. The columns were incubated for 30 minutes at 37°C then centrifuged at 893 rpm for 10 minutes. Reactions were graded according to manufacturer's directions. Random samples were selected to have serial dilutions made in 6% albumin and were tested against untreated and drug-treated cells. We chose at this time not to test SPRCA with drug-treated red blood cells as to the uncertainty of the cells' ability to coat the wells or for the drug to remain bonded to the cells. Therefore, with drug treated cells, we only compared the tube results to the gel system.

**RESULTS**

Forty-nine patient samples were tested with their previously identified putative drug. Tube testing results were taken from the patient historical files. Samples were tested in the same method as the original work: 28 were tested only in the presence of drug, 14 were tested only with drug treated cells and 7 were tested by both methods.

Drug–dependant antibodies identified in the presence of drug (IPOD)

Thirty-five samples were studied in the presence of the respective putative drug. All 35 samples tested had historical positive results when tested by tube IAT. There were 34 samples that tested positive in gel (97%); one sample was negative with cefotetan. Only 28 of 35 samples (80%) were positive using SPRCA. (Table 2) Negative control samples were non-reactive in both gel and SPRCA.

Reactivity using tubes and the gel system was greater as compared to SPRCA when tested with certain drugs. In the presence of diclofenac-metabolite, quinine and most notably with ceftriaxone there was greater disparity of reaction strength. Most samples (5/7) were 4+ in tubes in the presence of ceftriaxone, 6/7 were 4+ in gel while by SPRCA testing they were primarily in the 1-2+ range. With both pipercillin and Zosyn®, reactivity was weaker for some samples and non-detectable in others by SPRCA. (Table 3)

Drug-Dependant Antibodies Detected with Drug Treated Cells

Twenty-two samples were tested against drug-treated cells. Untreated cells were tested in parallel. With both tube testing and gel, 22/22 samples (100%) were positive. Negative control samples were non-reactive with drug treated cells. (Table 4) Samples tested against untreated cells had titers < 8. Titrations were performed on a random sampling of patients. Results with drug-treated cells in gel were

equal to or within one dilution less than or greater than their original studies performed on fresh sample. (Table 5)

Positive Indirect Antiglobulin Tests without Drug Added

Thirty-four samples were evaluated comparing tube, gel and SPRCA. By tube testing, 8/34 samples had a positive IAT in their initial serologic testing. Using gel, there were 9/34 samples positive without drug added. SPRCA testing resulted in 5/34 samples (15%) positive, one of which had not been reactive in tube. Comparing all 49 samples between tube testing and gel, there were 9 samples (18%) in tube that were reactive and 13 in gel (27%).

**DISCUSSION**

DIIHA, while uncommon, can be potentially very serious and even fatal. Drug dependent antibodies can be mistaken for warm or cold-type autoantibodies or alloantibody related to a delayed hemolytic transfusion reaction.[7,10,11] Testing for drug-dependent antibodies is determined by analyzing the ability of the suspected drug to coat red cells. In situations where there is no binding to the cells, then drugs in soluble form or metabolites of the drug may need to be present to detect the antibody. The gold standard for detection of these drug-dependent antibodies has been test tube based methods. Testing with drug-treated cells allows detection of antibodies for certain drug types. Penicillin, penicillin derivatives and cephalosporins generally will bind to red cells. However, pipercillin, a penicillin derivative and ceftriaxone, a cephalosporin, have been shown to react more readily in the presence of drug and in some cases do not react at all with drug coated cells.[3,12,13]

More transfusion services are using either microcolumn agglutination or solid phase for detection of antibodies. This study was designed to look at how these different methods would compare to tube testing for the detection of drug-dependent antibodies. Another concern was if either gel or SPRCA would show increased reactivity without drug added to the test system and possibly increase the probability of these antibodies being misinterpreted as autoimmune hemolytic anemia.

Forty-nine different patient samples that had previously been identified as having drug-dependent antibodies were thawed and retested in gel and SPRCA. The manufacturer's procedures were only slightly modified in this study, the variants being the addition of drug or albumin when testing for drug in soluble forms or using drug–treated cells in the testing procedure. There was a good correlation between

the results obtained by tube method and using gel IgG cards when testing in the presence of drug. The antibodies reacted in all samples tested by both tube and gel with the exception of one weakly reacting antibody. The strength of reactions was similar between these two methods. Stronger reactions (2-4+) in tubes were generally the same in gel; weaker reactions were comparable in grade between the two methods.

SPRCA was not as effective when testing in the presence of drug in this study. There were a number of non-reactive samples (8/36) as compared to the tube results. In addition, the strength of reactivity was less than seen with tubes or gel. One limitation of this study was that samples tested were taken from frozen stock which may have impacted results. However, the freeze/thaw manipulation of these samples did not appear to diminish reactivity in gel.

With drug-treated cells, the findings obtained using IgG gel cards were comparable to the tube results, similar to the original work by Salama et al.[5] All samples tested were positive by both methods. Drug-dependent antibody titers were closely matched between the two methods as well.

Of concern were gel results seen when no drug was added to the system. Salama et al.[5] did not indicate IAT results without drug added when a comparison between gel and tube testing was first reported. Both Arndt et al.[3] and Johnson et al.[4] have reported patients with drug-induced hemolytic anemia as having a positive IAT without drug added using tubes. Using gel in this study, there was an increased number of positive IATs without drug, a finding which might lead to a conclusion of autoimmune hemolytic anemia rather than drug-induced immune hemolytic anemia. The slight decrease in number of positive reactions with SPRCA may be due to reduced sensitivity without added drug present or the use of frozen samples.

The results of this study demonstrate that gel testing in the presence of drug and with drug-treated cells appears to be comparable to tube testing when drugs have been implicated as the cause of hemolytic anemia. SPRCA testing in the presence of drug may not detect some drug-dependent antibodies. Further studies need to be performed to assess testing with fresh versus frozen samples and the use of drug-treated cells in a solid phase system. SPRCA did not increase detection of antibodies without added drug as compared to test tube methods, however, there was an increase in the number of

samples reactive in gel without drug added. Finally, this study supports the importance of reviewing patient medication history, clinical history and transfusion history to avoid misdiagnosis.

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TABLE 1: Drugs used in Comparison of Tube, Gel, Gel/ficin and SPRCA Testing

| **Drug** | **Number** |
|---|---|
| Cephalosporins (23) | |
| Cefotetan | 15 |
| Cefoxitin | 1 |
| Ceftriaxone | 7 |
| Penicillin and/or penicillin derivatives (13) | |
| Ampicillin | 1 |
| Penicillin | 1 |
| Pipercillin | 6 |
| Nafcillin | 1 |
| Zosyn® (pipercillin/tazobactum) | 4 |
| NSAID (7) | |
| Diclofenac (urine metabolite) | 1 |
| Ibuprofin | 1 |
| Sulindac | 2 |
| Tolemetin | 3 |
| Quinine | 2 |
| Probenecid | 1 |
| Oxaliplatin | 3 |

TABLE 2: Patient Samples tested by Tube IAT, Gel and SPRCA in the presence of drug

| DIIHA Putative Drug | # of samples tested | # positive by Tube | # positive by Gel | # positive by SPRCA |
|---|---|---|---|---|
| Cefotetan | 7 | 7 | 6 | 6 |
| Ceftriaxone | 7 | 7 | 7 | 7 |
| Diclofenac (metabolite) | 1 | 1 | 1 | 1 |
| Ibuprofen | 1 | 1 | 1 | 0 |
| Oxaliplatin | 3 | 3 | 3 | 3 |
| Pipercillin | 4 | 4 | 4 | 2 |
| Probenecid | 1 | 1 | 1 | 1 |
| Quinine | 2 | 2 | 2 | 1 |
| Sulindac | 2 | 2 | 2 | 2 |
| Tolmetin | 3 | 3 | 3 | 3 |
| Zosyn® | 4 | 4 | 4 | 1 |
| Total | 35 | 35 | 34 | 28 |

TABLE 3: Comparison of reaction strength between Tube, Gel and SPRCA testing in the presence of ceftriaxone, pipercillin and Zosyn®.

| | Ceftriaxone (n=7) | | | Pipercillin (n=4) | | | Zosyn® (n=4) | | |
|---|---|---|---|---|---|---|---|---|---|
| Reaction Strength | Tube | Gel | SPRCA | Tube | Gel | SPRCA | Tube | Gel | SPRCA |
| 4+ | 6 | 6 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| 3+ | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| 2+ | 1 | 0 | 3 | 4 | 4 | 1 | 0 | 3 | 0 |
| 1+ | 0 | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 1 |
| wk | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 3 |

TABLE 4: Patient Samples tested with Untreated and Drug treated cells

| DIIHA Putative Drug | # of samples tested | # positive with drug treated cells – Tube | # positive with drug treated cells – Gel |
|---|---|---|---|
| Ampicillin | 1 | 1 | 1 |
| Cefotetan | 14 | 14 | 14 |
| Cefoxitin | 1 | 1 | 1 |
| Ceftriaxone | 1 | 1 | 1 |
| Nafcillin | 1 | 1 | 1 |
| Penicillin | 1 | 1 | 1 |
| Pipercillin | 3 | 3 | 3 |

TABLE 5: Titer comparisons between tube and gel with untreated and drug-treated red blood cells

| DIIHA Putative Drug | Titer with untreated cells Tube | Titer with drug treated cells – Tube | Titer with untreated cells Gel | Titer with drug treated cells – Gel |
|---|---|---|---|---|
| Ampicillin | 0 | 64 | 2 | 32 |
| Cefotetan(1) | 0 | 1,024 | 0 | 2,058 |
| Cefotetan(3) | 0 | 8,192 | 4 | 8,192 |
| Cefotetan(4) | 0 | 131,072 | 4 | >200 |
| Cefoxitin | 0 | 32,768 | 0 | 16,384 |
| Ceftriaxone | 0 | 32 | 0 | 64 |
| Nafcillin | 0 | 1024 | 0 | 512 |
| Penicillin | 0 | 8192 | 0 | 8192 |
| Pipercillin(1) | 1 | 32 | 1 | 32 |
| Pipercillin(2) | 1 | 32 | 1 | 32 |
| Pipercillin(3) | 0 | 8 | 0 | 4 |