Appendix.

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Abdel-Razeq SS, Buhimschi IA , Bahtiyar MO, Rosenberg VA, Dulay AT, Han CS, et al. A rapid indicator of intraamniotic inflammation and infection in "bloody tap" amniocenteses in women with symptoms of preterm labor. Obstet Gynecol 2010;116(2).

The authors provided this information as a supplement to their article.

I. Supplementary Methods

a) Indications for amniocentesis procedures, exclusion criteria, and definitions of preterm labor and preterm premature rupture of membranes (PROM)

All amniocenteses procedures were transabdominal and performed using the free hand technique under ultrasonographic guidance by maternal–fetal medicine specialists or fellows previously trained in performing this procedure. Gestational age was established based on the last menstrual period, an ultrasonographic examination, or both prior to 20 weeks of gestation. Preterm labor was defined as regular uterine contractions associated with advanced cervical dilatation or effacement in gestations before 37 weeks.[1] Membrane rupture was confirmed by well-established clinical criteria including vaginal speculum examination demonstrating pooling of amniotic fluid, a positive nitrazine paper, a positive ferning test result, or a combination of these. In instances where these test results were equivocal, the infusion of indigo carmine into the amniotic sac was used for definitive diagnosis. Clinical chorioamnionitis was diagnosed using one or more of the following: the presence of maternal fever (higher than 37.8 ºC), uterine tenderness, foul-smelling amniotic fluid, visualization of pus at the time of speculum exam, maternal tachycardia (more than 100 beats per min), or fetal tachycardia (more than160 beats per min).[2] The clinical decision regarding timing of delivery was made by the primary physician who was blinded to all research test results. Induction of labor or a surgical delivery was performed for clinical indications, such as amniotic fluid laboratory results indicative of intraamniotic infection, prolapsed umbilical cord, non-vertex presentation, abruption, or gestational age of 34 weeks or more in the context of preterm PROM.[3]

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b) Statistical analyses

Normality testing was performed using the Kolmogorov-Smirnov test. Statistical analysis was performed with Sigma Stat, version 2.03 (SPSS, http://www.spss.com) and MedCalc (http://www.medcalc.be) statistical softwares. Analyses included 1-way and 2-way analysis of variance (ANOVA), Kruskal-Wallis ANOVA, Chi-square analysis (in-log linear format for three-way contingency tables [http://faculty.vassar.edu/lowry/abc.html, accessed July 23, 2009]), and Spearman correlation.

In clinical practice, interpretation of the amniotic fluid white blood cell (WBC) count establishes diagnostic information relative to a cut-off. [4,5,6,7,8,9] As a result, two outcomes are possible: a positive or a negative test result. This approach is needed for ease and uniformity in interpretation of the results across patient populations, clinical settings, and institutions. We analyzed our data using several preset threshold values proposed in the literature for the amniotic fluid WBC count to confirm or exclude presence of inflammation or infection (10, 30, 50, 100 WBC/mm^3).[5,6,7,8,9] Furthermore, by using receiver operating curve (ROC) analysis, optimal diagnostic cut-off values were determined in our population with observed and corrected amniotic fluid WBC counts as continuous variables. Changes in diagnostic performance of amniotic fluid WBC count, which could potentially result from application of our correction formula, were evaluated using sensitivity, specificity, overall accuracy (ratio of cases correctly classified/total cases), graphic analysis of likelihood ratios (LRs) and matched-sample agreement tables.[10]

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The purpose of matched-sample agreement tables was to derive, in an analytical non-observational manner, the extent and subgroups where the correction most improves the diagnostic performances of the WBC count. For this, we constructed 2 x 2 contingency tables referencing for each case the binary result of the corrected against that of the observed WBC. This analysis was performed for all subgroups and for each WBC threshold as exemplified in Table 1.

Table 1. Layout of Matched Sample Agreement Tables for Diseased (eg Intraamniotic Inflammation, above) and Non-Diseased (below) in Bloody Tap Amniocentesis Specimens

Tests compared in sensitivity in BT samples		Observed WBC count +	Observed WBC count −	TOTAL
Corrected WBC count	+	a	b	a + b
	−	c	d	c + d
TOTAL		a + c	b + d	a + b + c + d (yes IAI* & yes BT) n=37

Tests compared in specificity in BT samples		Observed WBC count +	Observed WBC count −	TOTAL
Corrected WBC count	+	e	f	e + f
	−	g	h	g + h
TOTAL		e + g	f + h	e + f + g + h (no IAI* & yes BT) n=40

BT, bloody tap; WBC, white blood cell; a, true-positive by observed and true positive by corrected WBC count; b, true-positive by corrected and false-negative by observed WBC count; c, true-positive by observed and false negative by corrected WBC count; d, false-negative by observed and false-negative by corrected WBC count; IAI, intraamniotic inflammation; e, false-positive by observed and false-negative by observed WBC count; f, true-negative by observed and false-positive by corrected WBC count; g, false-positive by observed and true–negative by corrected WBC count; h, true-negative by observed and true–negative by corrected WBC count.

*An analogous analysis was done with diseased cases represented by those with a positive amniotic fluid culture result.

The McNemar's Chi square values (with Yates continuity correction) were calculated using the following formulas to compare sensitivities and specificities of the two tests. [10]

$$\textit{Chi square (sensitivity comparison)} = (|c - b| - 1)^2 / (c + b)$$

$$\textit{Chi square (specificity comparison)} = (|f - g| - 1)^2 / (f + g)$$

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Values above the Chi square critical levels of 3.841 (one degree of freedom), 6.635, and 10.827, indicated statistical significance at a level of $P<0.05$, $P<0.01$, and $P<0.001$, respectively.[10]

We next employed an analysis of the LRs based on the methods described by Biggerstaff BJ. [11] The confidence intervals for LRs were computed using the method proposed by Simel et al.[12] A graphic representation of the LRs analysis is presented in Figure 1.

Region	*LRs of any new test vs. R*	*Interpretation*
I.	Better +LR and -LR	New test is overall superior
II.	Worse +LR but better -LR	New test is inferior for confirming disease presence but superior at ruling it out
III.	Worse -LR and better +LR	New test is inferior at ruling out disease but superior at confirming its presence
IV.	Worse +LR and -LR	New test is overall inferior

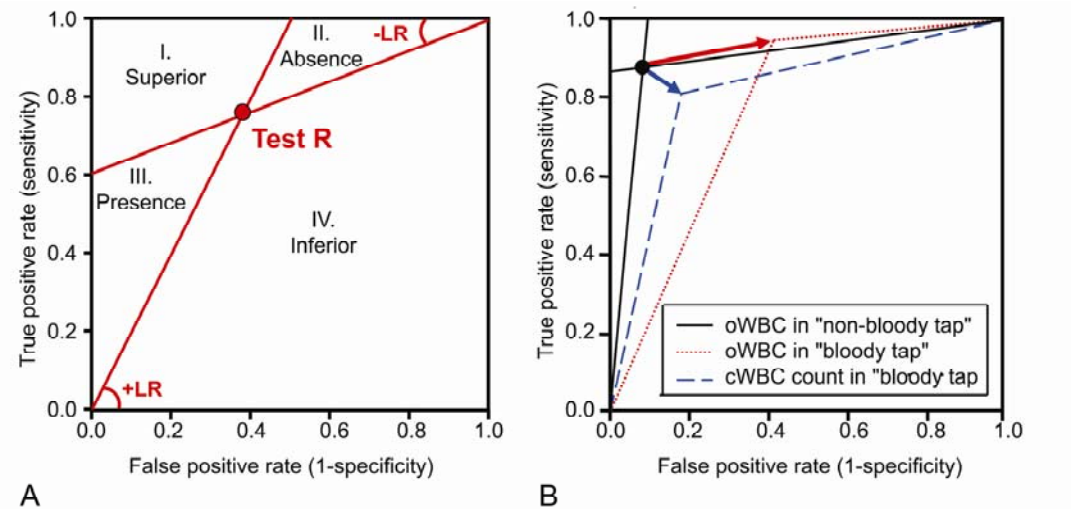

Figure 1. (A) Graphic method for interpretation of the likelihood ratios (LRs) based on the region of interest. **(B)** Graphic representation to aid comparison of the LRs for the observed white blood cell (oWBC) and corrected white blood cell (cWBC) count in "bloody" and "non-bloody" tap samples. The ascending line passing through (0,0) represents the slope of the +LRs of the observed and corrected WBC count. The horizontal lines passing through (1,1) represents the slope of the –LRs. The red vector is illustrative of the level of bias imposed by a "bloody tap" procedure. The blue vector represents an estimation of the proximity of the cWBC from the best possible scenario (oWBC in "non-bloody" tap samples).

Briefly, similar to standard ROC curve analysis, when plotting the area under the curve of a reference diagnostic test (R) with the true positive rate (sensitivity) on the vertical axis and the false-positive rate (1-specificity) on the horizontal axis, each point in this space can be represented as the intersection of two lines with slopes corresponding to +LR (ascending line) and –LR (horizontal line), respectively

(Figure 1A). As illustrated, four regions of comparison (I, II, III and IV) are then delineated. Determining in which region these lines intersect gives an overall appreciation of the practical utility of a new test (dashed lines) compared with the reference test (solid line). To quantify the clinical utility of WBC count correction in "bloody tap" amniotic fluid samples, we compared the vectorial distances between the points of intersection for the corrected compared with the observed WBC count (Figure 1B). This analysis allowed us to evaluate the diagnostic performance of the corrected WBC count in "bloody tap" samples, by observing its proximity to the diagnostic performance of the observed WBC count in "non-bloody tap" amniotic fluid sample, which was the best possible clinical scenario.

Agreement level calculations were performed using MedCalc software and interpreted as very good (kappa = 0.81-1.00), good (kappa = 0.61-0.80) moderate (kappa = 0.41-0.60), fair (kappa = 0.21-0.40) or poor (kappa <0.20).[13]

II. Supplementary Results

a) Flowcharts of enrolled patients

Using the cut-off of 1,000 RBC/mm^3, 22% (77/357) of the amniotic fluid samples investigated were classified as "bloody tap" specimens. Of these, 48% (37/77) had an MR score of 3-4. Forty additional "bloody tap" amniotic fluid samples were retrieved from women without intraamniotic inflammation. The stratification of cases by results of amniotic fluid cultures (Figure 2B) revealed a prevalence of intraamniotic infection of 27% (97/357). The higher prevalence of intraamniotic inflammation relative to infection was expected in the context of the previously demonstrated limitations of the amniotic

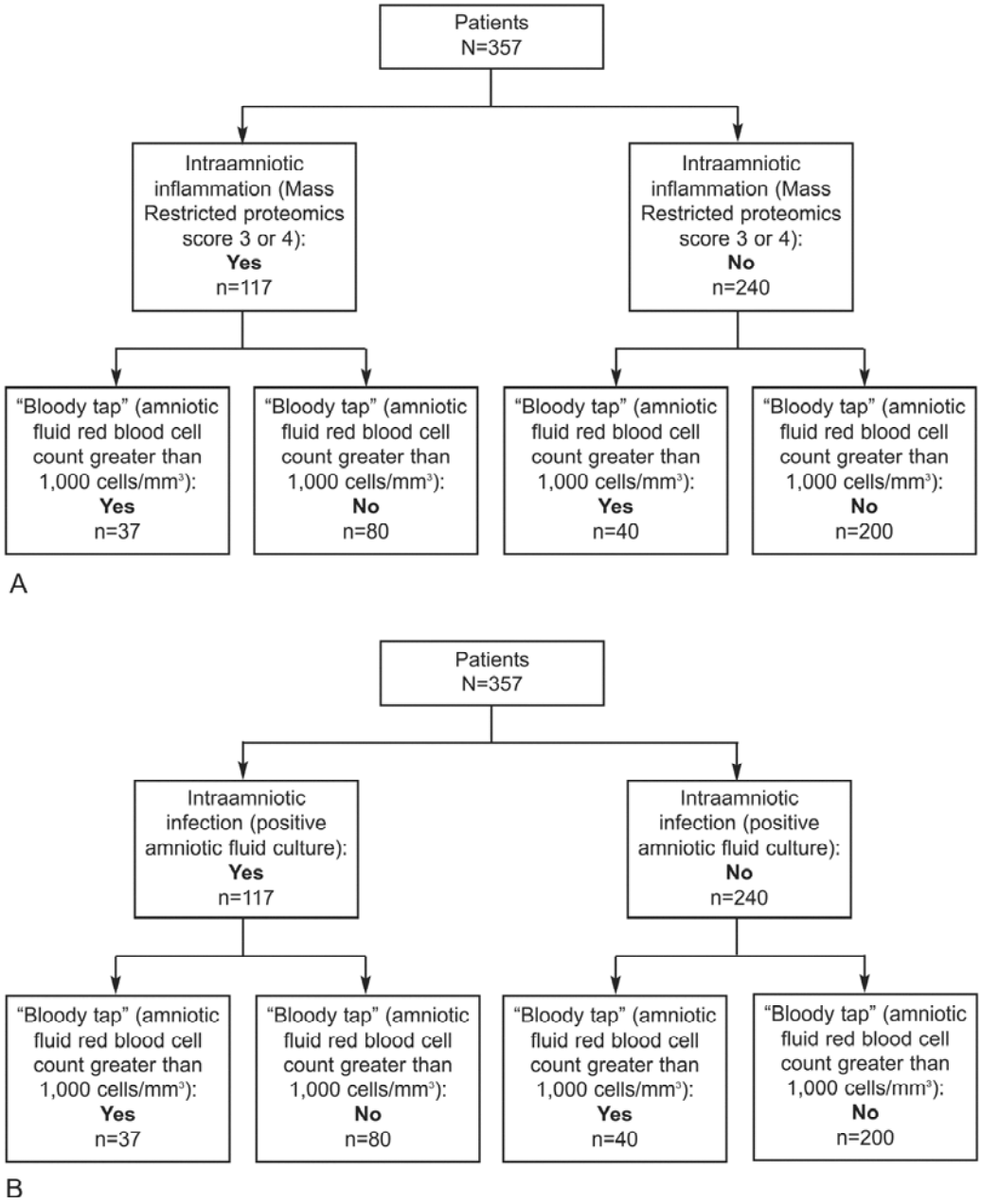

Figure 2 : Flowchart of patients distributed by **(A)** amniotic fluid inflammation (Mass restricted score 3-4) and "bloody tap" status and **(B)** by amniotic fluid infection (positive microbial cultures) and "bloody tap" status.

fluid cultures in identifying "true infection". Of the infected specimens, 32% (31/97) were classified as "bloody tap" as compared to only 17% (46/260) of the non-infected samples.

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b) Demographic, clinical, laboratory, and outcome characteristics of the patients

Table 2. Demographic, Clinical, and Outcome Characteristics of the Patients Who Provided Amniotic Fluid Samples

Variable	No IAI and No BT n = 200	Yes IAI and No BT n = 80	No IAI and Yes BT n = 40	Yes IAI and Yes BT n = 37	*P* value
Maternal characteristics at amniocentesis					
Age in years*	28 [22 – 32]	26 [23 – 33]	26 [22 – 32]	28 [23 – 34]	0.714
Gravidity*	2 [1 – 4]	2 [1 – 4]	2 [1 – 3]	3 [2 – 4]	0.708
Parity *	1 [0 – 1]	0 [0 – 1]	1 [0 – 1]	1 [0 – 2]	0.099
Race†					0.044
Caucasian	79 (39.5)	23 (28.8)	21 (52.5)	11 (29.7)	
African-American	57 (28.5)	30 (37.5)	7 (17.5)	17 (45.9)	
Hispanic	43 (21.5)	22 (27.5)	11 (27.5)	5 (13.5)	
Other	21 (10.5)	5 (6.3)	1 (2.5)	4 (10.8)	
Gestational age in weeks†	29.1 [25.1 – 31.8]	26.2 [24.2 – 29.4]	29.7 [26.8 – 32.4]	25.4 [23.6 – 30.3]	0.002
Ruptured membranes†	67 (34)	33 (41)	25 (63)	27 (73)	<0.001
Clinical chorioamnionitis†	9 (5)	11 (14)	0 (0)	5 (14)	0.005
Uterine contractions†	89 (45)	45 (56)	9 (23)	17 (46)	0.006
Cervical dilatation*	1 [0 – 2]	2 [1 – 4]	1 [0 – 2]	1 [0 – 2]	<0.001
Anterior placental location	92 (46)	33 (41)	27 (68)	22 (59)	0.021
Outcome characteristics					
Gestational age at delivery in weeks*	32.5 [28.1 – 35.5]	26.3 [24.8 – 30.0]	31.8 [29.3 – 34.1]	25.5 [24.1 - 30.3]	<0.001
Amniocentesis-to-delivery in days *	7.9 [1.3 – 39.8]	0.36 [0.2 – 0.8]	6.1 [1.9 – 27.3]	0.61 [0.2 – 1.6]	<0.001
Birthweight in grams*	1,969 [1,220 – 2,540]	1,000 [780 – 1,470]	1,995 [1,430 – 2,179]	812 [705 – 1,467]	<0.001
Cesarean delivery†	72 (36)	25 (31)	11 (28)	16 (43)	0.445
Apgar score at 1 minute*	8 [5 – 9]	7 [3 – 8]	8 [5 – 9]	6 [2 – 8]	<0.001
Apgar score at 5 minutes*	9 [8 – 9]	8 [6 – 9]	9 [8 – 9]	8 [4 – 9]	<0.001

IAI, intraamniotic inflammation as depicted by a mass-restricted score of 3 or 4; BT, "bloody tap" as depicted by an amniotic fluid red blood cell count of more than 1,000 cells/mm^3.

* Data presented as median [interquartile range] and analyzed by Kruskal-Wallis 1-way analysis of variance.

†Data presented as n (%) and analyzed by Chi square tests.

Women of African-American descent were diagnosed with intraamniotic inflammation more frequently compared to Caucasian subjects and those of other races (Chi-square, *P*=0.014), independent of RBC contamination of the amniotic fluid (*P*=0.902). We determined that women with intraamniotic inflammation were of shorter gestational age at amniocentesis irrespective of the "bloody tap" status (2-way ANOVA inflammation, *P*=0.002; "bloody tap," *P*=0.362). In our cohort, a "bloody tap" sample was more frequently retrieved in women with preterm PROM independent of

inflammation (three-way Chi-square corrected by inflammation, $P<0.001$). However, clinical chorioamnionitis was more often encountered in women with intraamniotic inflammation independent of the amniotic fluid RBC count (3-way Chi-square corrected by "bloody tap," $P<0.001$). Although a "bloody tap" was seen more frequently in women with a cervical dilation less than 2 cm and in women without uterine contractions, these differences disappeared when correcting for the status of the membranes at amniocentesis. An anterior placenta was associated more often with a "bloody tap" procedure, a finding independent of the presence or absence of intraamniotic inflammation (three-way Chi-square corrected by inflammation, $P=0.008$). In a multivariate logistic regression model, preterm PROM, anterior placenta, and intraamniotic inflammation, were independent predictors of a "bloody tap" specimen (preterm PROM odds ratio (OR), 3.2 [1.8-5.6], $P<0.001$; anterior placenta OR, 2.1 [1.2-3.7], $P=0.009$; inflammation OR, 2.3 [1.3-4.1], $P=0.003$). We determined that presence of intraamniotic inflammation, but not of a "bloody tap", significantly affected pregnancy outcomes related to preterm birth, such as gestational age at delivery, amniocentesis-to-delivery interval, birthweight and Apgar scores (two-way ANOVA, $P< 0.001$).

The results of the amniotic fluid and maternal hematological analysis are presented in Table 3. Both "bloody tap" and inflammation effect the amniotic fluid WBC count with significant interaction between the two variables (two-way ANOVA, "bloody tap" $P=0.002$; inflammation $P<0.001$; interaction $P=0.002$). Conversely, amniotic fluid glucose concentration, lactate dehydrogenase activity and interleukin (IL)-6 levels were related only to the presence of intraamniotic inflammation, but not to the "bloody tap" status of the sample ($P<0.001$). A positive amniotic fluid Gram stain and a positive microbial culture result were more frequently recorded in the setting of inflammation, independent of the number of amniotic fluid RBC (3-way Chi-square corrected by "bloody tap", $P<0.001$). The

Table 3. Demographic, Clinical, and Outcome Characteristics of the Patients Who Provided Amniotic Fluid Samples

Variable	No IAI and No BT n = 200	Yes IAI and No BT n = 80	No IAI and Yes BT n = 40	Yes IAI and Yes BT n = 37	*P* value
Amniotic fluid					
WBC count (cells/mm^3)*	4 [1 – 10]	679 [105 – 1,730]	18 [9 – 58]	778 [197 – 2,062]	<0.001
RBC count (cells/mm^3)*	22 [4 – 153]	67 [11 – 245]	5,290 [1,810 – 16,250]	6,000 [1,405 – 59,600]	<0.001
Glucose (mg/dL)*	30 [21 – 39]	5 [2 – 17]	26 [19 – 44]	2 [2 – 12]	<0.001
LDH(U/L)*	162 [110 – 231]	750 [475 – 1,422]	150 [115 – 266]	626 [441 – 1,580]	<0.001
Inteleukin-6, ng/mL	0.52 [0.2 – 1.6]	29.2 [9.0 – 64.8]	0.32 [0.2 – 2.2]	15.5 [9.1 – 48.4]	<0.001
Positive Gram stain†	11 (6)	41 (51)	4 (10)	16 (76)	<0.001
Positive cultures†	11 (6)	55 (69)	8 (20)	23 (62)	<0.001
Maternal peripheral blood					
WBC count x 10^3 (cells/mm^3)*	12 [10 – 15]	14 [11 –.17]	11 [9 – 15]	15 [12 – 20]	<0.001
Leukocytosis greater than 15,000 WBCs/mm†	44 (22)	32 (40)	10 (25)	18 (49)	<0.001
Hematocrit, (percent)*	34.6 [32.3 – 36.5]	34.0 [31.7 – 35.5]	34.5 [32.8 – 36.8]	33.5 [29.3 – 35.9]	0.030
MCV (fL)*	88 [84 – 92]	89 [86 – 93]	89 [86 – 94]	89 [86 – 91]	0.142
RBC count, x 10^6 (cells/mm^3)*	3.9 [3.6 – 4.2]	3.8 [3.6 – 4.1]	3.9 [3.5 – 4.3]	3.8 [3.4 – 4.1]	0.051

IAI, intraamniotic inflammation as depicted by a mass restricted score of 3 or 4; BT, "bloody tap" as depicted by an amniotic fluid red blood cell count greater than 1,000 cells/mm^3;WBC, white blood cells; RBC, red blood cells; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; fL femtoliters.

* Data presented as median [interquartile range] and analyzed by Kruskal-Wallis 1-way analysis of variance.

†Data presented as n (%) and analyzed by Chi square tests.

analysis of maternal peripheral blood variables demonstrated that women with proven intraamniotic inflammation had elevated WBC counts, a higher frequency of leukocytosis (WBC count of 15,000 cells/mm^3 or more) and a lower hematocrit, independent of the "bloody tap" condition of the amniotic fluid (two-way ANOVA, $P<0.001$ for all variables). There were no differences in mean corpuscular volume among groups.

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