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A point of care test for the determination of amniotic fluid interleukin-6 and the chemokine CXCL-10/IP-10

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Abstract

Objective—Intra-amniotic inflammation is a mechanism of disease implicated in preterm labor, preterm PROM, cervical insufficiency, a short cervix, and idiopathic vaginal bleeding. Determination of interleukin (IL)-6 with immunoassays has been proven for more than 2 decades to be an excellent method for the detection of intra-amniotic inflammation. However, assessment of IL-6 for this indication has been based on immunoassays which are not clinically available, and this has been an obstacle for the implementation of this test in clinical practice. It is now possible to obtain results within 20 minutes with a point of care test which requires minimal laboratory support. This test is based on lateral flow-based immunoassay. The objective of this study was to compare amniotic fluid (AF) IL-6 and interferon-γ –inducible protein 10 (IP-10 or CXCL-10) concentrations determined using lateral flow-based immunoassay or point of care (POC) test and standard enzyme-linked immunosorbent assay (ELISA) techniques.

Material and methods—AF samples were collected from patients with singleton gestations and symptoms of preterm labor (n=20). AF IL-6 and IP-10 concentrations were determined by lateral flow-based immunoassay and ELISA. Intra-amniotic inflammation was defined as AF IL-6 > 2.6 ng/ml. AF IL-6 and IP-10 concentrations between two assays were compared.

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Results—1) Lateral flow-based immunoassay point of care AF IL-6 and IP-10 test results were strongly correlated with concentrations of this cytokine/chemokine determined by ELISA (Spearman's ρ =0.92 and 0.83, respectively, both $p < 0.0001$); 2) AF IL-6 concentrations determined by the lateral flow-based immunoassay test were, on average, 30% lower than those determined by ELISA, and the median difference was statistically significant ($p < 0.0001$); and 3) in contrast, AF IP-10 concentrations determined by the lateral flow-based immunoassay test were, on average, only 7% lower than those determined by ELISA, and the median difference was not statistically significant (p =0.81).

Conclusion—Amniotic fluid IL-6 and IP-10 concentrations determined using a lateral flow-based immunoassay point of care are strongly correlated with concentrations determined by conventional ELISA. This justifies further studies about the diagnostic indices and predictive values of this point of care test.

Keywords

Amniocentesis; acute chorioamnionitis; chronic chorioamnionitis; lateral flow-based immunoassay; intraamniotic inflammation/infection; interferon-γ –inducible protein 10 (IP-10); MIAC; prematurity; sterile inflammation

Introduction

Intra-amniotic infection and inflammation are causally linked to preterm birth [1-13], which is the leading cause of perinatal morbidity and mortality [14-35]. The gold standard for the diagnosis of intra-amniotic infection has relied on cultivation of bacteria in the amniotic fluid (AF) [36-61]. Amniotic fluid culture detects microbial invasion of the amniotic cavity (MIAC) in approximately 10% of patients with preterm labor and intact membrane [37,38,41,43,47,50,62-80], 30% of patients with preterm prelabor rupture of membrane (PROM) [40,46,49,53,56,60,81-85] and about 50% of pregnant women with acute cervical insufficiency [86-89]. Patients with a short cervix [90,91] or those with idiopathic vaginal bleeding [92] have a frequency of MIAC of approximately 9%.

Several studies have now shown that patients with intra-amniotic inflammation have adverse pregnancy and neonatal outcome, even if there is no evidence of microorganisms in the amniotic cavity, suggesting that the diagnosis of intra-amniotic inflammation is key [57,58,91,93,94].

Multiple biomarkers for the diagnosis of intra-amniotic inflammation have been used, including determinations of cytokines [68,73,94-114], chemokines [115-121], matrix-degrading enzymes [122-129], and other inflammatory mediators [130-140]. Parameters have ranged from a simple amniotic fluid white blood cell (WBC) count [49,70,71,141] to the determination of analytes with immunoassays or bioassays [interleukin-8 (IL-8), chemotaxis] [115,116,142,143]. Amniotic fluid concentrations of IL-6 have been reported by several investigators to be informative and reliable indicators of the prognosis of patients at risk for preterm delivery [49,61,100,144-148]. IL-6 determinations have been performed in most studies, using research immunoassays not available for clinical management. Often, clinical laboratories in hospitals send samples to reference laboratories, and results are not

available for days. What is necessary is a rapid test that can inform clinical decision-making. Several investigators have reported a rapid IL-6 test for the diagnosis of adult [149] and neonatal sepsis [150,151], as well as inflammation of the cerebrospinal fluid [152]. Kacerovsky et al. have recently reported the use of a point of care (POC) test for IL-6 in preterm prelabor rupture of membranes (PROM), which uses lateral-flow immunoassays [113]. Other investigators have used determination of IL-6 in vaginal fluid [153,154] as a point of care test.

We have described a new form of intra-amniotic inflammation that occurs in patients with preterm labor, and which is characterized by an elevation of amniotic fluid CXCL-10, rather than IL-6 [155]. This form of intra-amniotic inflammation is associated with chronic chorioamnionitis, anti-fetal HLA maternal sensitization [156], and is thought to represent maternal anti-fetal rejection [157]. Therefore, it may be important to have a rapid test to identify an elevation of CXCL-10 in amniotic fluid.

The objective of this study was to determine the relationship between the amniotic fluid concentrations of IL-6 and CXCL-10 (different markers of intra-amniotic inflammation) determined by lateral-flow immunoassays and conventional enzyme-linked immunosorbent assay (ELISA).

Methods

Study design and participants

A cross-sectional study was conducted by searching the clinical database and bank of biologic samples of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of *Eunice Kennedy Shriver* National Institutes of Child Health and Human Development (NICHD) (Detroit, MI), to identify patients with a diagnosis of spontaneous preterm labor. Patients were included if they met the following criteria: 1) had a singleton gestation; 2) presented with preterm labor; and 3) had a transabdominal amniocentesis performed between 20 and 35 weeks of gestation with microbiologic studies. Patients were excluded from the study if they had: 1) rupture of the chorioamniotic membranes occurred before AF collection; 2) a chromosomal or structural fetal anomaly. In order to have a wide range of IL-6 for the assay comparison, we selected 3 groups of patients in which: 1) preterm labor delivered at term; 2) preterm labor delivered preterm without intra-amniotic infection; and 3) preterm labor and delivered preterm with intra-amniotic infection. All patients provided written informed consent; the use of biologic specimens and clinical data for research purposes was approved by the Institutional Review Boards of NICHD and Wayne State University.

Clinical Definitions

Preterm labor was diagnosed by the presence of at least two regular uterine contractions every 10 minutes in associated with cervical changes in patients with a gestational age between 20 and 36 6/7 weeks which led to preterm delivery (defined as birth prior to the 37th week of gestation). Acute histologic chorioamnionitis was diagnosed based on the presence of neutrophils in the chorionic plate and/or chorioamniotic membranes [158-160]. Intra-

amniotic inflammation was diagnosed when IL-6 AF concentration was > 2.6 ng/ml [57]. MIAC was defined according to the results of AF culture. Intra-amniotic infection was defined as a combination of MIAC with intra-amniotic inflammation.

Biological samples and analysis

Patients with preterm labor and intact membranes who underwent transabdominal ultrasound-guided amniocentesis for evaluating possible MIAC (within the standard of care at Hutzel Women's Hospital, Detroit, Michigan) were eligible for the study. AF was transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital mycoplasmas. Evaluation of white blood cell (WBC) count, glucose concentration, and Gram stain of AF were also performed shortly after collection. AF not required for clinical assessment was centrifuged for 10 min at 4°C shortly after and stored at −70°C until analysis. The presence of intra-amniotic infection inflammation was assessed by determination of AF interleukin-6 (IL-6) concentration by ELISA. AF IL-6 concentrations were determined for research purposes, and such results were not used in patient management.

Analysis of amniotic fluid samples for IL-6 and IP-10 concentrations

AF IL-6 and IP-10 concentrations (ng/ml) were determined both by ELISA and lateral flow-based immunoassay point of care (POC) test. For ELISA, AF concentrations of IL-6 and IP-10 were determined by immunoassays obtained from R&D Systems (Minneapolis, MN, USA). The details of these immunoassays and their performance have been previously described [49,57,96,101,142,161-170]. The point of care determination of AF IL-6 and IP-10 concentrations (ng/ml) was performed using a lateral flow-based immunoassay point of care (POC) test (Milenia QuickLine® IL-6; Milenia Biotec, Bad Nauheim, Germany). Briefly, 100 μL of AF were transferred to the test unit and after 15 min of incubation, 50 μL of buffer was added. In the first step, IL-6 present in the sample binds to a monoclonal anti-human IL-6 antibody (Ab) conjugated to gold particles. The complex of IL-6 and the conjugate continues to migrate through the analytical membrane. A test line and a control line are printed on this membrane. The test line is coated with a second anti-IL-6 Ab directed against a different epitope than the conjugate Ab. Once the complex of IL-6 and the conjugate passes the test line, it forms a sandwich with the coated IL-6 Ab. A band becomes visible PicoScan® densitometry system (Milenia Biotec, Bad Nauheim, Germany). This system measures the intensity of the test band and calculates concentrations according to standard curve. Results are typically available after 20 minutes and can be interpreted both visually and by densitometry. The assay time, volume and other assay characteristics for each method are shown in Table 1.

Statistical Analysis

The Kolmogorov-Smirnov test was used to assess normality of arithmetic data distributions. The Kruskal-Wallis test for comparisons and the Mann-Whitney U test were used to make comparisons among and between groups for arithmetic variables. Chi-square was used for comparisons of categorical variables. Correlation between AF IL-6 concentrations determined by lateral flow-based immunoassay point of care (POC) and ELISA was assessed using Spearman's correlation coefficients. Bland-Altman plots were constructed to

assess between-assay agreement. General linear models were fit to determine coefficients of determination (r^2). Statistical analysis was performed using SPSS 19 (IBM Corp, Armonk, NY, USA) and SAS 9.4 (Cary, NC, USA). A p value < 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics of the study population are displayed in Table 2. Ten of the 20 patients with preterm labor delivered at term. Three of the remaining ten patients who delivered preterm had intra-amniotic infection, and each was delivered at < 32 weeks of gestation. Organisms identified in the AF of these three patients included Gram negative bacilli, *Ureaplasma urealyticum* and *Gemella Morbillorum*.

Correlation among amniotic fluid IL-6 concentrations determined by point of care and standard ELISA techniques

All of the samples had IL-6 concentrations above the lowest limit of detection (sensitivity) for both assays. Two samples from patients with microorganisms in the amniotic cavity had AF IL-6 concentrations that were above the standard threshold of the lateral flow-based immunoassay POC test (concentrations were high and precise calculation would have needed dilution of the sample and a new assay). Excluding these two patients, AF IL-6 concentrations determined using ELISA and the lateral flow-based immunoassay POC test were strongly correlated (Spearman ρ =0.92; p= <0.0001). The Bland-Altman plot analysis (Figure 1) shows that AF IL-6 concentrations determined by the POC test were, on average, approximately 30% lower than those determined by ELISA (Sign rank test for median difference, p < 0.0001). Figure 2 shows that this was a systematic observation, as it was consistent among patients. Overall, 92% of the variability in AF IL-6 as determined by ELISA was explained by the lateral flow-based immunoassay POC test results (r^2 0.92, β=21.9, standard error=1.66, p <0.0001) (Figure 3).

Correlation among amniotic fluid IP-10 concentrations determined by point of care and standard ELISA techniques

AF IP-10 concentrations determined using the POC test were strongly correlated with those determined by ELISA (Spearman ρ =0.83; p= <0.0001). The Bland-Altman plot for these two assays (Figure 4) shows that IP-10 concentrations were only approximately 7% lower, on average, than those determined by ELISA (Sign rank test for median difference, p = 0.81). Figure 5 demonstrated the actual concentrations in all patients between these 2 techniques. Overall, 80% of the variation in AF IP-10 concentrations determined by ELISA was explained by the lateral flow-based immunoassay POC test results (r^2 0.80, β=0.67, standard error=0.08, p <0.0001, Figure 6).

Discussion

Principal findings of the study

1) The lateral flow-based immunoassay point of care amniotic fluid IL-6 and IP-10 test results were strongly correlated with concentrations determined by ELISA; 2) AF IL-6

concentrations determined by the lateral flow-based immunoassay were, on average, 30% lower than those determined by ELISA, and the median difference between assays was statistically significant ($p < 0.0001$); and 3) in contrast, AF IP-10 concentrations determined by the lateral flow-based immunoassay POC test were, on average, only 7% lower than those determined by ELISA, and the median difference between assays was not statistically significant ($p = 0.81$). These findings justify the need for further studies about the diagnostic indices and predictive values of these point of care tests.

Point of care test for amniotic fluid IL-6—A large body of evidence indicates that patients with intra-amniotic inflammation are at greater risk for impending preterm delivery and other adverse outcomes, even when the results of AF culture are negative for microorganisms [57,58,91,93,94]. Our group has previously shown that an AF IL-6 concentration ≥ 11.3 ng/ml has a sensitivity of 93% and a specificity of 92% for the identification of positive AF culture, and also that it is predictive of preterm delivery, amniocentesis-to-delivery interval and neonatal morbidity and mortality [50]. Subsequently, the AF IL-6 cut-off of 2.6 ng/ml was proposed to diagnose intra-amniotic inflammation that is associated with a shorter amniocentesis-to-delivery interval, and also with significantly higher risks of adverse neonatal outcomes, *even among patients with a negative AF culture* [57]. The cut-off of 2.6 ng/ml has been extensively used in the literature by other investigators to identify those at risk for adverse pregnancy outcome in patients with preterm labor and intact membranes [129,171-173].

Patients with an elevated IL-6 but no evidence of microorganisms with cultivation methods and molecular microbiologic techniques are considered to have sterile intra-amniotic inflammation, and are at high risk for adverse pregnancy outcome in the context of preterm labor with intact membranes [58,59], preterm PROM [60], and a short cervix [91]. Therefore, the detection of an elevated IL-6 seems to be clinically important.

In the present study, we demonstrated that AF IL-6 concentrations measured using a lateral flow-based immunoassay POC test were strongly correlated with those determined using the conventional ELISA technique. These observations are consistent with findings of studies involving adult [149]/neonatal sepsis [150,151] and subarachnoid hemorrhage [152], which also reported that IL-6 concentrations measured with a point of care test correlated strongly with those measured by ELISA.

In addition, prior studies with this point of care test for IL-6 have shown that results correlate with the microbial burden of *Ureaplasma sp* in AF from patients with preterm PROM [113], and that a cut-off of 1,000 pg/ml had a 50% sensitivity and a 95% specificity for the identification of MIAC or both MIAC and acute histological chorioamnionitis in these patients [113]. A high negative predictive value (97%) has also been reported for a point of care vaginal fluid IL-6 concentration in identifying intra-amniotic inflammation in patients with rupture of membranes [154].

The ELISA method was chosen for the comparison since this technique is currently considered the gold standard for IL-6 determination. However, ELISA results are not practical, because they are not available in time for clinical decision-making. In contrast, the

point of care IL-6 assay allows IL-6 determinations within 20 minutes and might be useful in some institutions that are not able to perform IL-6 ELISA rapidly. The assay characteristics are different; the point of care test can measure IL-6 between 0.05–10 ng/ml for AF IL-6, while the IL-6 ELISA has a range between 0.003 ng/ml and 0.3 ng/ml. Although the assay sensitivity of the latter is much higher, repeated assays with further sample dilutions would take more time to obtain valid assay values. The standard threshold of IL-6 point of care test is 10 ng/ml, however, from a clinical perspective this threshold is acceptable, since most patients with intra-amniotic inflammation and adverse perinatal outcomes have AF IL-6 concentrations > 2.6 ng/ml. Moreover, there would be no difference in the clinical management of patients with an IL-6 +/− 10.

Amniotic fluid IP-10 and preterm labor: a point of care test for the identification of intra-amniotic inflammation—We have previously reported that a subset of patients with preterm labor have a different form of intra-amniotic inflammation that is associated with elevated AF IP-10 concentrations [155] and a higher prevalence of chronic chorioamnionitis, the most common placental lesion in late spontaneous preterm birth. This lesion is characterized by maternal T-cell infiltration of the chorion laeve and amnion, and resembles allograft rejection [157]. We have reported that chronic chorioamnionitis is associated with anti-fetal HLA maternal sensitization [156] and complement deposition in umbilical vein endothelium [155], which is associated with a novel form of fetal systemic inflammation characterized by overexpression of T cell chemokines, such as IP-10 or CXCL-10 [174]. An elevation of mid-trimester AF IP-10 concentration is associated with late (but not early) spontaneous preterm delivery [170]. Thus, it is possible that patients with an elevated CXCL-10/IP-10 are at increased risk for spontaneous preterm delivery. Similar to IL-6, ELISA is currently the gold standard for assessment of AF IP-10. Our observations in this study support that AF IP-10 determined by the POC test is strongly correlated with that determined by ELISA. Further studies are warranted to determine the diagnostic performance of IP-10 by the POC test in the identification of adverse obstetrical outcomes.

A point of care test for the identification of intra-amniotic inflammation—A simple definition for point of care testing is: “Diagnostic testing performed at or near the site of patients care” [175]. This testing is designed to be quick, readily available, accurate, and useful for clinical decision making and considered valuable to increase clinical efficiency and improve medical and economic outcomes [175-178]. The AF lateral flow-based immunoassay point of care (POC) fulfills most of the criteria proposed to assess an optimal POC, namely: 1) simple testing methodology; 2) rapid availability of the results (up to 20 minutes); 3) easy interpretation of the results; 4) low maintenance, because the kit can be stored at room temperature; 5) strong correlation with standard laboratory procedures (ELISA); and 6) low cost, because there is no need for capital equipment and the market price can be driven by need. Larger studies are needed to determine whether other criteria pertaining to clinical utility and cost-benefit are satisfied.

Conclusions

A POC test using lateral-flow based immunoassay for the determination of IL-6 and IP-10 concentrations in amniotic fluid yields results that are strongly correlated with those determined by ELISA. Further studies are warranted to determine the diagnostic and prognostic performance of these tests in clinical obstetrics.

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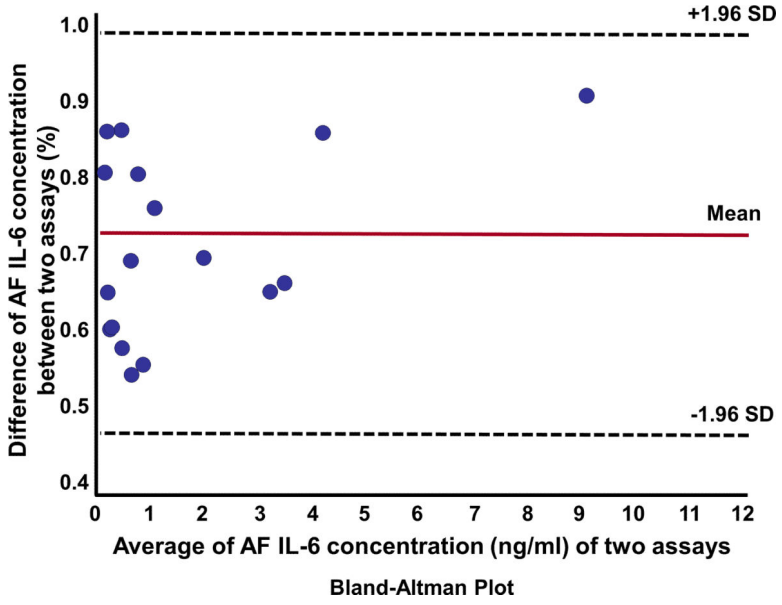

Figure 1.

Bland-Altman plot: direct comparison between enzyme-linked immunosorbent assay (ELISA) and lateral flow-based immunoassay point of care (POC) amniotic fluid (AF) interleukin-6 (IL-6) techniques. AF IL-6 concentrations from three patients with preterm labor with microbial invasion of the amniotic cavity (MIAC) were excluded. SD=standard deviation.

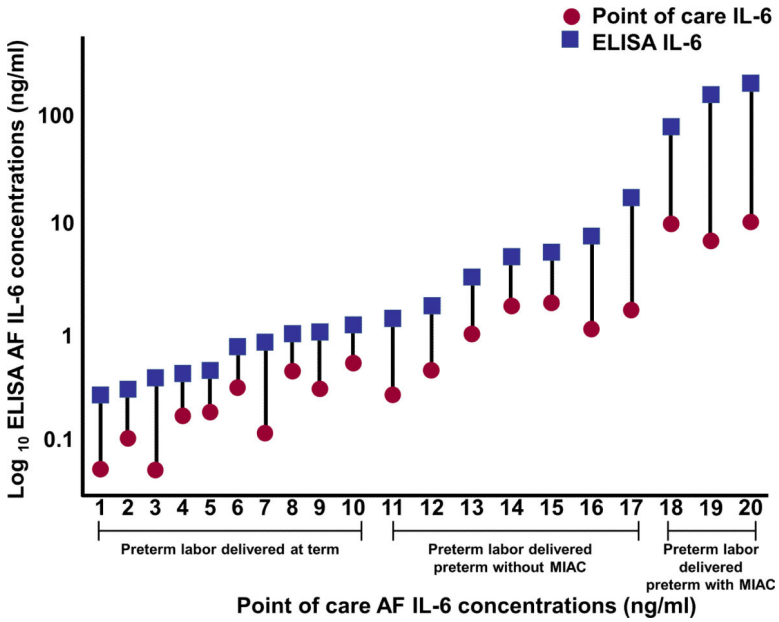

Figure 2.

Amniotic fluid (AF) concentrations of interleukin-6 (IL-6) determined by enzyme-linked immunosorbent assay (ELISA) (square) and a lateral flow-based immunoassay point of care (POC) test (circle) of patients with preterm labor. Lateral flow-based immunoassay point of care (POC) AF IL-6 concentrations were significantly lower than those of ELISA in every pair samples.

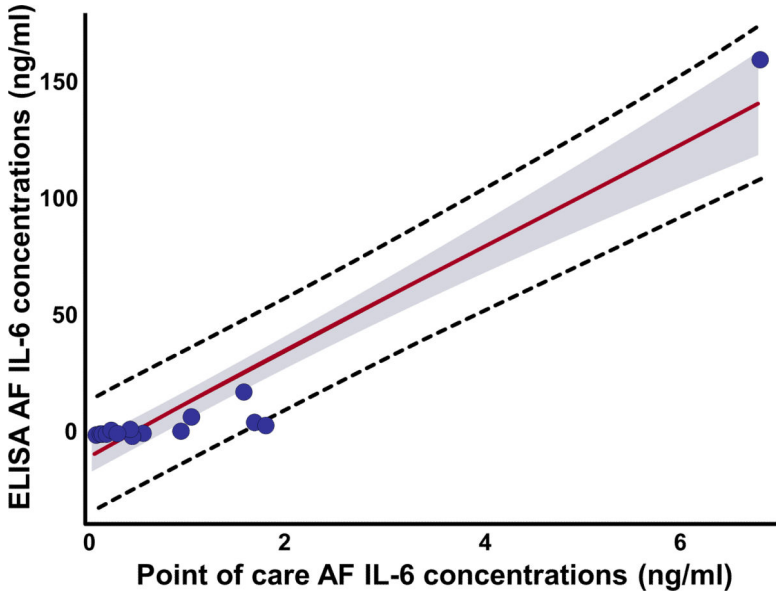

Figure 3.

Amniotic fluid (AF) interleukin-6 (IL-6) scatter diagram with linear regression line (red line). Dashed line indicates 95% confidence interval. AF IL-6 concentrations from three patients with preterm labor with microbial invasion of the amniotic cavity (MIAC) were excluded.

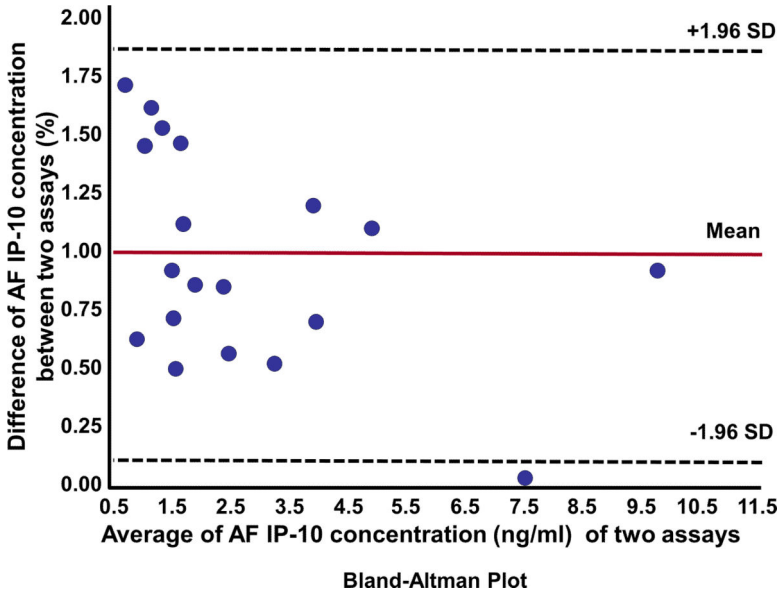

Figure 4.

Bland-Altman plot: direct comparison between enzyme-linked immunosorbent assay (ELISA) and lateral flow-based immunoassay point of care (POC) amniotic fluid (AF) interferon-γ –inducible protein 10 (IP-10 or CXCL 10) techniques. SD=standard deviation.

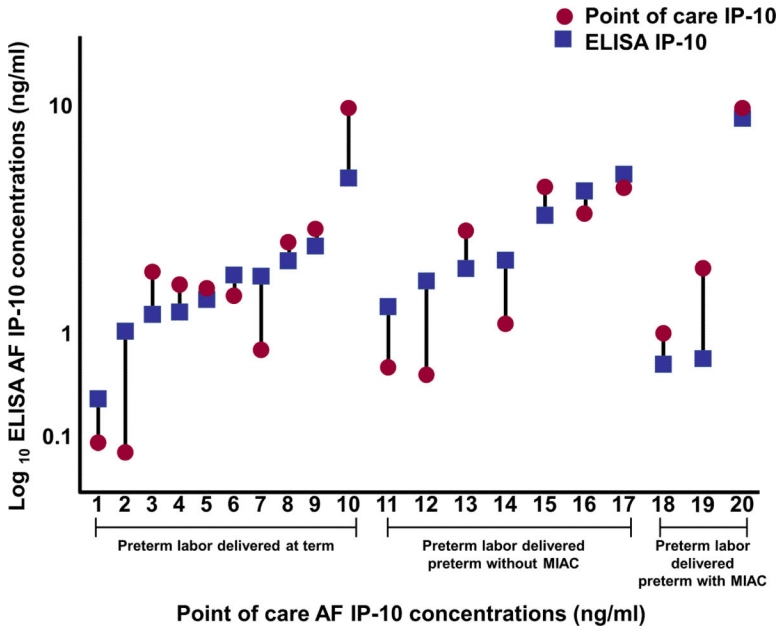

Figure 5.

Amniotic fluid (AF) concentrations of interferon-γ –inducible protein 10 (IP-10 or CXCL-10) determined by enzyme-linked immunosorbent assay (ELISA) (square) and lateral flow-based immunoassay point of care (POC) test (circle) of patients with preterm labor.

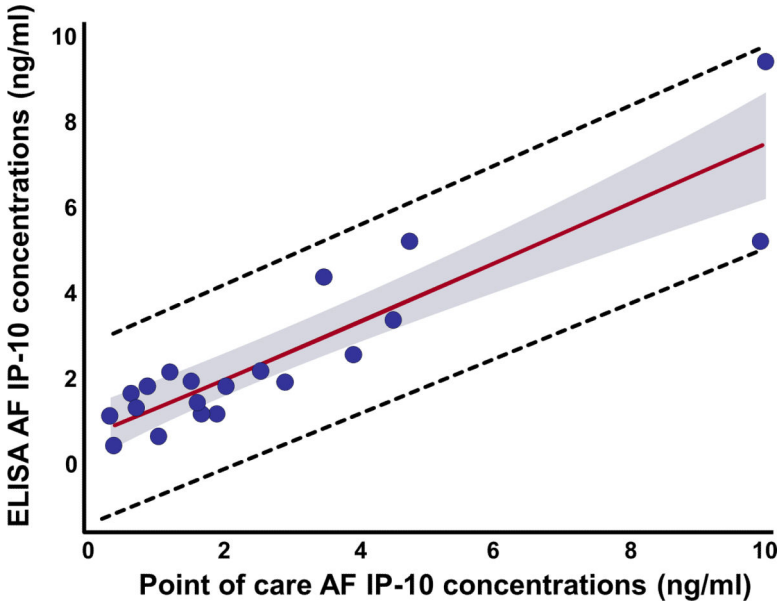

Figure 6.

Amniotic fluid (AF) interferon-γ –inducible protein 10 (IP-10 or CXCL-10) scatter diagram with linear regression line (red line). Dashed line indicates 95% confidence interval.

Table 1

Comparison of assay characteristics between ELISA and lateral flow-based immunoassay point of care test (POC)

	Interleukin-6 ELISA	Interleukin-6 point of care test	IP-10 ELISA	IP-10 point of care test
Assay time	5 hours	20 minutes	5 hours	20 minutes
Sample volume	12 μL	100 μL	10 μL	100 μL
Sensitivity	< 0.70 pg/ml	50 pg/ml	1.67 pg/ml	100 pg/ml
Range of detection	3.13-300 pg/ml	50-10,000 pg/ml	7.8-500 pg/ml	50-10,000 pg/ml
Intra-assay coefficients of variation	2.9%	12.1%	2.8%	12.1%
Inter-assay coefficients of variation	2.8%	15.5%	3.5%	15.5%

IP-10 or CXCL-10: interferon-γ –inducible protein 10

ELISA: enzyme-linked immunosorbent assay

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Table 2

Characteristics of study populations

	Preterm labor (n=20)
Age (years)	22 (20-25)
Parity	40% (8/20)
Body mass index (kg/m^2)	23.3 (21.1-32.3)
Gestational age at amniocentesis (weeks)	30.5 (28.3-31)
Gestational age at delivery (weeks)	26.3 (31.5-39.2)
Birthweight (grams)	2617(1,718-3,002)
Amniotic fluid glucose (mg/dl)	25 (17-30)
Amniotic fluid white blood cell (cell/mm)	0 (0-5)
Amniotic fluid IL-6 ELISA (ng/ml)	1.2 (0.5-6.9)
Amniotic fluid IL-6 point of care test (ng/ml)	0.4 (0.2-1.6)
Amniotic fluid IP-10 ELISA (ng/ml)	1.8 (0.9-3.8)
Amniotic fluid IP-10 point of care test (ng/ml)	1.8 (1.3-3.2)
Placental lesions associated with amniotic fluid infection[^]	42% (8/19)

Data presented as % (N) or median (interquartile range), IL: interleukin, IP: interferon-γ –inducible protein 10 or CXCL 10, MIAC: microbial invasion of the amniotic cavity microbial; ELISA: enzyme-linked immunosorbent assay

[^]placental lesions associated with amniotic fluid infection include acute chorioamnionitis and funisitis.