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ARTICLE *in* JOURNAL OF IMMUNOLOGICAL METHODS · OCTOBER 2015

Impact Factor: 1.82 · DOI: 10.1016/j.jim.2015.09.004

Journal of Immunological Methods 427 (2015) 36–41

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Magnetic bead fluorescent immunoassay for the rapid detection of the novel inflammation marker YKL40 at the point-of-care

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ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form 21 August 2015
Accepted 23 September 2015
Available online 3 October 2015

Keywords:
Immunoassay
Point of care
Magnetic beads
Fluorescence
Pneumonia
YKL40

ABSTRACT

Pneumonia is one of the leading causes of death worldwide. We present a magnetic bead fluorescent sandwich immunoassay that allows rapid serum measurement of the novel inflammation marker YKL40 (CHI3L1) at the point of care (POC) that could aid pneumonia diagnosis. The magnetic beads serve as the solid phase for separation of YKL40 from serum. The readout is performed using a small and robust fluorescence reader, which detects the turnover of a fluorescent substrate. The assay procedure, from sample addition to data retrieval, consists of three steps and is performed in less than 20 min. The presented assay has a linear range from 3 to 111 ng/mL, with a limit of detection of 2.9 ng/mL. The average recoveries were found between 101 and 111%. The developed method was applied in sera from healthy subjects (n = 14; c(YKL40) = 50 ± 49 ng/mL) and from pneumonia patients (n = 14; c(YKL40) = 333.6 ± 225 ng/mL). The elevated YKL40 concentrations in pneumonia-diseased patients are in good agreement with previously published data. The POC-ready device provides a simple immunoassay that could help to optimize pneumonia inflammation diagnostics in low-resource settings.

1. Introduction

Pneumonia is an acute infection caused by various bacteria and virus species. The disease is characterized by augmented bronchoalveolar liquid and pus congesting the respiratory tract, resulting in shortness of breath (Evertsen et al., 2010; Gennis et al., 1989; Ruiz-González et al., 1999). However, even with many diagnostic parameters for clinical examination (e.g. coughing, chills, bronchial breathing, dulled percussion and high temperature), there is high uncertainty in physicians' diagnoses (Wipf et al., 1999). This variation in symptom presentation has been found to significantly delay the time in which patients receive the necessary antibiotics (Metersky et al., 2006). The gold standard for diagnosing pneumonia is to combine the data from clinical symptoms with chest imaging techniques (Gennis et al., 1989), blood culture of microbial organisms (Akter et al., 2014), and serum biomarker measurements, such as the acute-phase parameter C-reactive protein (CRP) (Florin and Ambroggio, 2014; Requejo and Cocoza, 2003). Yet, some emergency departments still fail to achieve higher than 75% diagnosic accuracy for pneumonia (Sikka et al., 2012).

YKL40 (Chitinase-3-like protein 1) is a 40 kDa glycoprotein that plays a role in many processes involving angiogenesis, inflammation, and tissue remodeling (Johansen, 2006). It has been shown to be chitin-binding, but shows no chitinase activity (Renkema et al., 1998). It interacts with numerous proteins and carbohydrates, but the exact physiological function in humans is still unknown (Prakash et al., 2013). Recently, there is increasing evidence that YKL40 may play a crucial role in angiogenesis (Shao, 2013) and in mucin 5A production of bronchial epithelial cells (Liu et al., 2013). Several studies have shown that YKL40 is a relevant biomarker in pulmonary inflammatory disease, particularly for pneumonia and asthma (Korthagen et al., 2014; Kronborg et al., 2002; Létuvé et al., 2008; Nordenbaek et al., 1999; Ober et al., 2008). For instance, patients with *Streptococcus pneumoniae* related pneumonia had eight to ten times elevated serum YKL40 at hospitalization compared to healthy controls (Kronborg et al., 2002; Nordenbaek et al., 1999). Furthermore, serum YKL-40 was already found to be associated with higher all-cause mortality in a cohort of 493 COPD patients in Denmark (Holmgaard et al., 2013).

In human endotoxemia, a condition initiated by endotoxin derived from Gram-negative pathogens, YKL40 has been shown to increase after at least 2 h, while CRP increased not before 8 h after admission of an inflammatory stimulus (Johansen et al., 2005). Although YKL40 has also been found to increase in serum of patients with cancer, asthma,

Abbreviations: 4PL, 4-parameter logistic model; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CV, coefficient of variation; DDAO, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one); DDAO-P, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) phosphate; EDC, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide; ELISA, enzyme linked immunosorbent assay; HSA, human serum albumin; LED, light-emitting diode; LOD, limit of detection; LOQ, limit of quantification; mAB, monoclonal primary antibody; MB-FIA, magnetic bead fluorescence immunoassay; NHS, N-hydroxysuccinimide; SD, standard deviation; pAB, polyclonal secondary antibody; PCR, polymerase chain reaction; POC, point-of-care; SP, streptavidin-conjugated alkaline phosphatase; YKL40 (CHI3L1), chitinase3-like1.

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http://dx.doi.org/10.1016/j.jim.2015.09.004
0022-1759/

and arthritis-related diseases, patients with pneumonia have even higher YKL40 serum concentrations, on average, than that of the other diseases (Kim et al., 2012) — making it a candidate marker for pneumonia and respiratory disease detection.

Given the fact that more than 99% of pneumonia deaths occur in low-income regions around the world, such as Sub-Saharan Africa and South Asia (Rudan et al., 2011), a cheap and reliable technology, which has the ability to quickly detect biomarker levels, is sorely needed (Tong, 2013). Conventional diagnostic immunoassays are time and resource consuming and not easily applicable to a point-of-care (POC) environment, where rapid measurement and robust, transportable devices are required (Luppa et al., 2005). Thus, in order to increase the speed of diagnosing pneumonia, no matter the area in which the diagnosis takes place, new, robust diagnostic tests and indicative biomarkers are needed.

Magnetic bead-based immunoassays are, today, widely used in different application areas, because the implementation of micro-to-nanometer scale magnetic beads as a solid support potentially reduces the required incubation time from hours to minutes (Sista et al., 2008). However, the complexity of processing and detection often make them unapplicable to low resource settings (Tekin and Gijs, 2013).

Our aim was to establish a rapid and simple magnetic bead-based immunoassay for the detection of the novel biomarker YKL40 in an innovative POC-ready device for assistance in the diagnosis of pneumonia. We applied the ESEQuant Tube Scanner, a small fluorescence measurement system that utilizes a fluorescence detector based on LED and light filter technology, to enable the measurement of fluorescence in PCR-tubes even without an external power source (Njiru, 2012). The device has already been successfully used in fluorescence-based nucleic-acid tesing in low resource settings (Abd El Wahed et al., 2013; Xia et al., 2014).

2. Materials and methods

2.1. Reagents

CHI3L1 (YKL40) protein and monoclonal primary (mAB) and biotinylated polyclonal secondary (pAB) antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Streptavidin-conjugated alkaline phosphatase (SP) and human serum albumin (HSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Carboxyl-modified magnetic beads with an average diameter of 600 nm (range 500–900 nm) were from MagnaMedics Diagnostics BV (Geleen, The Netherlands). The magnetic separator was acquired from Chemicell (Berlin, Germany). 7-hydroxy-9 H-(1,3-dichloro-9,9-dimethyl-acridin-2-one) phosphate (DDAO-P) was obtained from AAT Bioquest (Sunnyvale, CA, USA, Art. No. 11629). 1-Ethyl-3-(3-dimethylaminopropyl)carbo-diimide (EDC) and N-hydroxysuccinimide (NHS) were from General Electric (Fairfield, CT, USA). All other laboratory chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and are of analytical grade. The washing buffer was prepared with 50 mM Tris–HCl-buffer (pH 7.4), 150 mM NaCl, and 0,05%(*v*/*v*) Tween 20. Substrate solution was prepared with 100 mM Tris (pH 9,5), 100 mM NaCl, 5 mM MgCl2, and 1 μM DDAO-P.

2.2. Apparatus

All experiments were performed using the above mentioned ESEQuant Tube Scanner device. Excitation and emission wavelengths have been adjusted to 625 and 680 nm, respectively, to fit the spectral properties of the fluorescent DDAO. The ESEQuant Tube Scanner uses a confocal microfluorescence reader, which is small enough to be automatically positioned under each tube. The measurement is repeated every 20 s, leading to the recording of the time course of substrate turnover. Emitted light intensity (Int) was recorded over time in mV, the first derivative of the signal is then given as dInt/dt (mV/min). The first derivative was used to eliminate the background signal.

2.3. Preparation of magnetic beads

500 μL (5 mg) of carboxyl-modified beads were washed twice in 500 μL of dH_2O. An NHS-ester was formed by the application of 750 μL 480 mM EDC and 10 mM NHS for 15 min at 29°C under agitation using a thermo mixer. Washing with 500 μL dH_2O removed excess reagents. The coupling reaction was carried out by addition of 200 μL (100 μg) of monoclonal YKL40 capture antibody in PBS for 1 h at 29°C under agitation. The supernatant was checked for residual soluble antibody using a standard Bradford assay, obtained from BIO-RAD (Richmond, CA, USA). Absence of protein in the supernatant was interpreted as successful coupling. The modified beads were blocked and stored for at least 30 min in dilution buffer before use.

2.4. Statistics

We used the Kruskal–Wallis test to determine the differences between the subject groups. The data was displayed as mean ± standard deviation (SD). Values of $p < 0.05$ were regarded as statistically significant. The limit of detection (LOD) (ng/mL) was defined as ([blank value] + 3 × SD of blank value) and limit of quantification (LOQ) (ng/mL) as ([blank value] + 6 × SD of blank value). Statistical analysis was completed with the R statistical computing software (R Core Team, 2013) and plotting performed with Origin, version 8.5 (Origin Lab, Northampton, MA, USA). The % recovery was calculated as (obtained conc./nominal conc.) × 100. The acceptable range and the molecular mechanisms for percentage recovery are explained elsewhere (Westgard, 2008).

2.5. Human serum samples

Healthy blood donor sera were obtained from the Bavarian Red Cross, and patient sera were collected at the Klinikum rechts der Isar. Only patients with radiologically confirmed pneumonia were included. The study was approved by the local ethics committee, and all included subjects gave informed consent. YKL40-depleted serum was prepared by incubation of 1 mL pooled control serum with 70 μL of antibody-conjugated magnetic beads for 30 min. The supernatant was checked for residual signals; signals equal to buffer were interpreted as successful YKL40 depletion (data not shown). Serum samples were diluted 1/5 (*v*/*v*) using 50 mM Tris–HCl (pH 7.4), 5% (*w*/*v*) HSA (Dilution Buffer).

3. Results

3.1. Immunoassay procedure

The general *M*agnetic *B*ead *F*luorescent *I*mmuno*A*ssay (MB-FIA) procedure consists of three steps (Fig. 1A). First, 10 μL of reagent mix – containing capture monoclonal antibody-modified magnetic beads and a detection polyclonal antibody-SP complex – was aliquoted into each tube of an 8-tube PCR Strip. The assay reaction is then initiated by the addition of 40 μL of sample to each tube. The mixture is incubated under gentle agitation (e.g. on a vertical rotating device). After incubation, the tube strips are placed in a magnetic separator for 2 min, to attract and gather the magnetic beads, and the supernatant is decanted. After washing, 50 μL substrate solution is added. If the analyte YKL40 was present in the sample solution, the SP bound to the detection antibody cleaves the phosphate group from DDAO-P (Fig. 1B). Fig. 2 shows an example of assay readout data. Because DDAO-P and DDAO emit fluorescent signals at different wavelengths, the YKL40-dependent turnover can be measured directly by the fluorometer (Fig. 2A). The signals are analyzed using the first derivative of the DDAO fluorescence measurements, produced by each tube after 1 min, thereby eliminating the background signal (Fig. 2B).

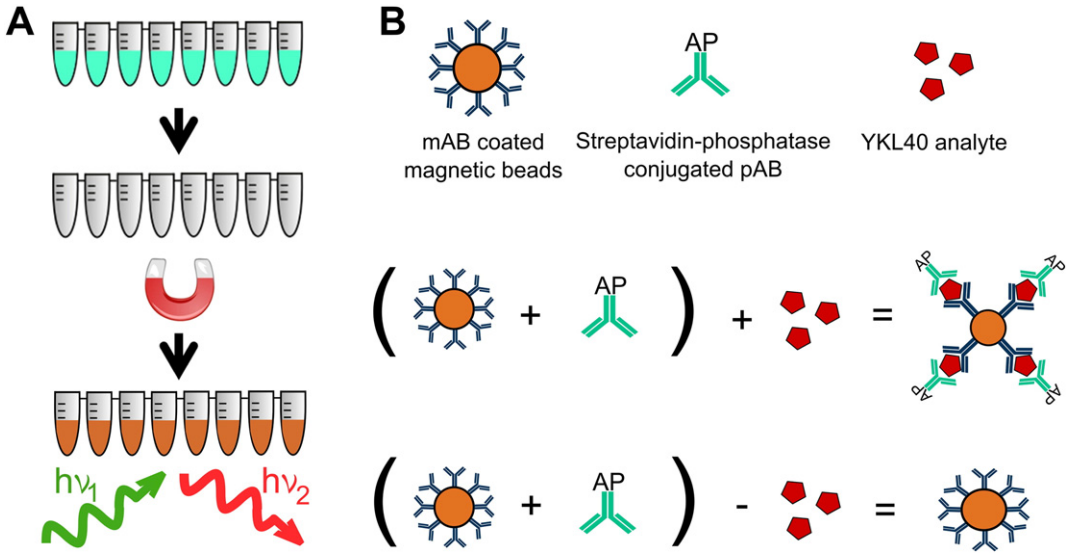

Fig. 1. A: The assay procedure consists of three steps including magnetic separation; the detection takes place in a small portable device. B: When antigen is present, an immune complex sandwich forms, allowing the phosphatase to be attached to the magnetic beads.

3.2. Immunoassay development

3.2.1. Phosphatase concentration

The signals produced in the magnetic bead sandwich assay are generated by the turnover of DDAO-P to DDAO by the SP complex attached to the biotinylated detection pAB. Thus, we considered how many SP complexes can bind to each detection antibody, in order to maximize signal output. The DDAO-P turnover rate was measured after adjusting the concentration of the SP complex to 2.5 nM, 5 nM, 15 nM, 25 nM, and 50 nM, while the pAB concentration was kept constant at 7 nM (Fig. 3A). We found that the signals did not rise at SP complex concentrations above 15 nM, and we concluded that approximately two SP complexes bound to each detection polyclonal antibody in the magnetic bead suspension.

3.2.2. Primary and secondary antibody concentrations

In the presented assay format, concentration of the secondary antibody plays an important role for obtaining high and specific signals. The signals related to the ratio of capture to detection antibodies were tested at different concentrations: 2:1, 3:1, 4:1. Increasing the concentration of the detection antibody directly increased absolute signals (Fig. 3B). We determined that the 2:1 ratio was optimal in maintaining high absolute signals without using excess reagents in what is designed to be a low-cost diagnostic resource.

3.2.3. Assay optimization and performance data

Further experimental parameters were optimized (Table 1). Fig. 4 shows the calibration curve for YKL40 in 1/5 (*v*/*v*) diluted YKL40-depleted serum using the optimized assay procedure. The LOD and LOQ for the optimized assay were found to be 2.9 ng/mL and 5.1 ng/mL, respectively, with coefficients of variation (CV) ranging between 0.85% and 12.3% at each point. The graph presents a linear range from 3 ng/mL to 111 ng/mL ($R^2 = 0.98$). Recovery experiments were carried out using three different control sera spiked with 10, 25, and 50 ng/mL recombinant YKL40 protein; for dilution experiments, three different patient sera were diluted to 1/5, 1/10, and 1/20. Recoveries were between 101 and 115% and, thus, were sufficient for the analysis of serum samples (Table 2). The inter- and intraassay-CV were determined to be 5.5 ($n = 6$) and 8.3% ($n = 8$) for a sample containing 333 ng/mL YKL40.

3.2.4. Human sera measurement

In order to test the applicability of the magnetic bead assay as a POC device, we analyzed a preliminary set of sera from 14 healthy blood donors and 14 patients with radiologically confirmed pneumonia. As shown in Fig. 5, there is a clear distinction ($p < 0.01$) between the signals obtained from healthy subjects and patients with pneumonia. The mean calculated serum concentration of patients with pneumonia was 334 ng/mL (median 300 ng/mL, range

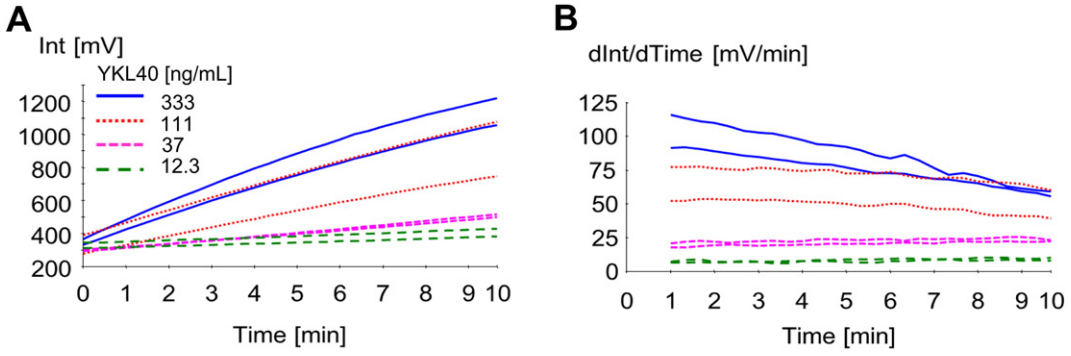

Fig. 2. A: Exemplary assay readout. After conducting the assay, the substrate turnover can be measured directly via fluorescence detection; each line represents one tube. All measurements are conducted in duplicate. The signals are dependent on the actual concentration of YKL40 in the sample. B: The first derivative eliminates the background signal and thus gives a more reliable and faster readout. Therefore, the YKL40 concentrations can be distinguished more clearly.

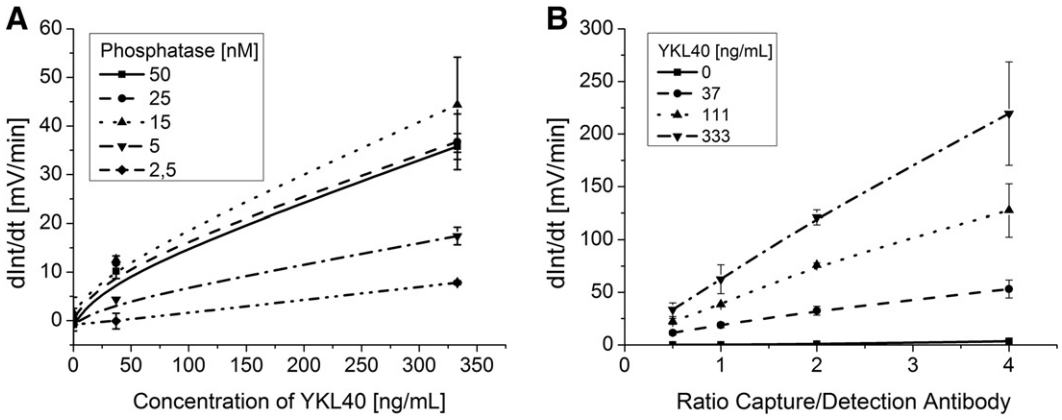

Fig. 3. A: Different concentrations of streptavidin-phosphatase, compared to detection pABs, were measured to determine the optimal ratio. Streptavidin-phosphatase concentrations of 2.5 nM, 5 nM, 15 nM, 25 nM, and 50 nM were measured, while detection pAB concentration remained at 7 nM. B: Optimal ratio of capture to detection pABs. Different ratios of capture-to-detection antibodies were implemented to discover which amount would achieve the highest signals. Starting at 7 nM:7 nM, we measured the ratios at 1:1, 2:1, 3:1, and 4:1.

Table 1
Summary of optimized parameters and specifications.

Parameter	Value
No. washing steps	2
Phosphatase/detection antibody	15:7 (30 nM/14 nM)
Capture/detection antibody	2:1 (28 nM/14 nM)
Incubation temperature (°C)	23
Detection temperature (°C)	37
Incubation time (min)	10
Limit of detection (ng/mL)	2.9
Limit of quantification (ng/mL)	5.1
Total assay time (min)	17

107–821 ng/mL, SD 225 ng/mL), while serum samples from healthy donors averaged at 50 ng/mL (median 35 ng/mL, range 9–199 ng/mL, SD 49 ng/mL).

4. Discussion

The present study shows the fast and sensitive detection of the novel pneumonia biomarker YKL40 in serum, using a magnetic bead-based fluorescence immunoassay in a POC-like device. The assay protocol consists of 1) using YKL40 monoclonal capture antibodies conjugated to magnetic beads and polyclonal detection antibody-streptavidin phosphatase complexes to extract YKL40 from the sample, 2) washing the magnetic beads to reduce background noise, and 3) adding DDAO-P and quantifying the phosphatase-driven turnover to DDAO. After establishing the sandwich assay, we adjusted the protocol in order to optimize and configure the assay to the required measurement range.

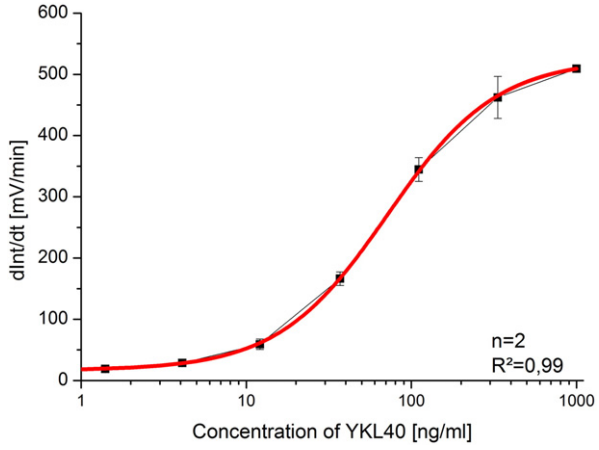

Fig. 4. Calibration curve for the optimized assay. YKL40 concentrations of 0; 1.4; 4.1; 12.1; 37; 111; 333; and 1000 ng/mL were measured in diluted YKL40-depleted serum. A 4-parameter logistic model (4PL) was fitted to the data (parameters see supplemental material).

However, when attempting to measure extremely high YKL40 values, over 1000 ng/mL, the saturation of assay reagents with analyte would produce a signal lower than what was to be expected, which is a phenomenon well-known to analysts (Burtis et al., 2012). Thus, we pre-diluted the serum samples to one-fifth of the original volume to ensure that the YKL40 concentrations could be accurately measured. Because the presented assay has a LOD of 2.9 ng/mL, we were able to measure YKL40 levels in samples from both healthy and diseased patients. The total assay time was below 20 min using the 3-step protocol.

We noticed that the absolute fluorescence signals showed considerable variability within the first minutes of substrate turnover. Therefore, we used the first order derivative of the signals produced from the turnover over the span of 1 min, which leads to the elimination of this initial variation and provides a faster and more reliable reading.

Assay performance data, such as sensitivity, linearity, and precision have been validated. As per the FDA bioanalytical method guidelines, which allow for a variability of up to ±20%, the variability of our developed immunoassay, 101–115%, is well within the recommended range. Furthermore, we have confirmed by additional experiments that YKL40 was depleted in the human sera (Supplemental Table 1) as the reading of the assay conducted for the detection of YKL40 in the human serum is equal to that in dilution buffer alone. The results indicated that the developed YKL40 assay could be employed in the analysis of this parameter satisfactorily. YKL40 has been reported to be approximately 40 ng/mL in healthy blood serum (Bojesen et al., 2011), whereas the concentration of YKL40 in an individual subject with pneumonia can reach values above 1000 ng/mL (Kronborg et al., 2002). Measuring a preliminary set of serum samples in the magnetic bead assay, we found concentrations comparable to these findings. Considering the upper pathophysiological concentration range of YKL40 where the concentration can be above 1000 ng/mL, we have to take a higher dilution of the analyte sample together with the dilution employed in our

Table 2
Left: spike recovery in three different control sera. Right: dilution series in three different patient sera.

Spiked concentration (ng/mL)	Average recovery (%) (n = 3)	CV (%)	Dilution factor	Average recovery (%) (n = 3)	CV (%)
10	101.0	23.7	2	115.1	7.7
25	107.6	25.5	4	113.5	9.4
50	111.5	31.1			

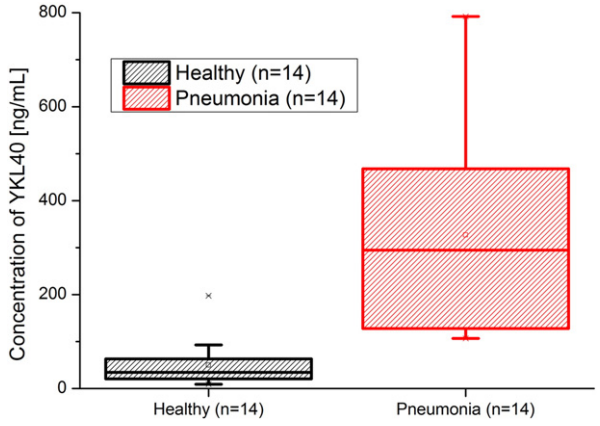

Fig. 5. Using the MB-FIA, YKL40 levels were measured for 14 sera taken from pneumonia patients and 14 from healthy subjects. The signals resulting from the YKL40 measurements are shown in a box-and-whisker plot: top and bottom of box equate to the 75th and 25th percentile, respectively; middle line represents the median; top and bottom whiskers represent highest and lowest, non-outlier, levels, respectively.

immunoassay so that the concentration of YKL40 falls within the linear range of the developed immunoassay. The comparison of various ELISA procedures and their efficacies in the precise determination of YKL-40 is subject of our future investigation.

The MB-FIA presents several advantages over current immunoassays. These benefits include short assay run time (<20 min) and potential accessibility for healthcare providers in low-resource settings. Existing immunoassay measurement systems, such as ELISAs, that can be used to analyze YKL40 levels may take several hours for analysis and quantification. Also, an ELISA plate reader is often not easily to be transported between hospitals and clinics in rural areas of developing countries. The ESEQuant Tube Scanner, however, is able to run on a replaceable and portable battery system, relinquishing the need for a power supply needed for ELISA devices. The combination of reducing time needed to retrieve vital information and enabling ease in availability of YKL40 testing make the presented MB-FIA a novel biomarker detection system for low-income regions secluded from major healthcare centers.

Also, diagnostic information about disease progression of chronic obstructive pulmonary disease (COPD) by peripheral serum biomarkers is mandatory for patients who suffer frequent acute exacerbations, leading to severe breathlessness, respiratory failure, and death (Holmgaard et al., 2013). There is clinical need for robust, yet easily accessible, serum biomarkers for disease monitoring in order to optimise personalized medicine in patients with COPD (Shaw et al., 2014). Thus, our fluorescence immunoassay will be adopted in further clinical studies to explore its diagnostic value for such an attempt.

The establishment of this fluorescence-based MB-FIA is a proof of concept that we can accurately and rapidly receive protein biomarker data using antibodies bound to magnetic beads in a POC-ready device, providing a simple alternative immunoassay that could help optimize pneumonia diagnosis in low resource settings. The key features of the presented assay are a rapid fluorescence measurement below 20 min, a detection limit that corresponds well to the lower reference range of this inflammation serum marker, and usage of an inexpensive and robust device.

Acknowledgments/funding

We like to thank Anita Schreiegg for the excellent technical assistance. The study was part of the [RES]check project. We gratefully acknowledge the financial support of the BMBF (Grant no. #16SV5433K).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2015.09.004.

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