Article

Design of Multiplex Lateral Flow Tests: A Case Study for Simultaneous Detection of Three Antibiotics

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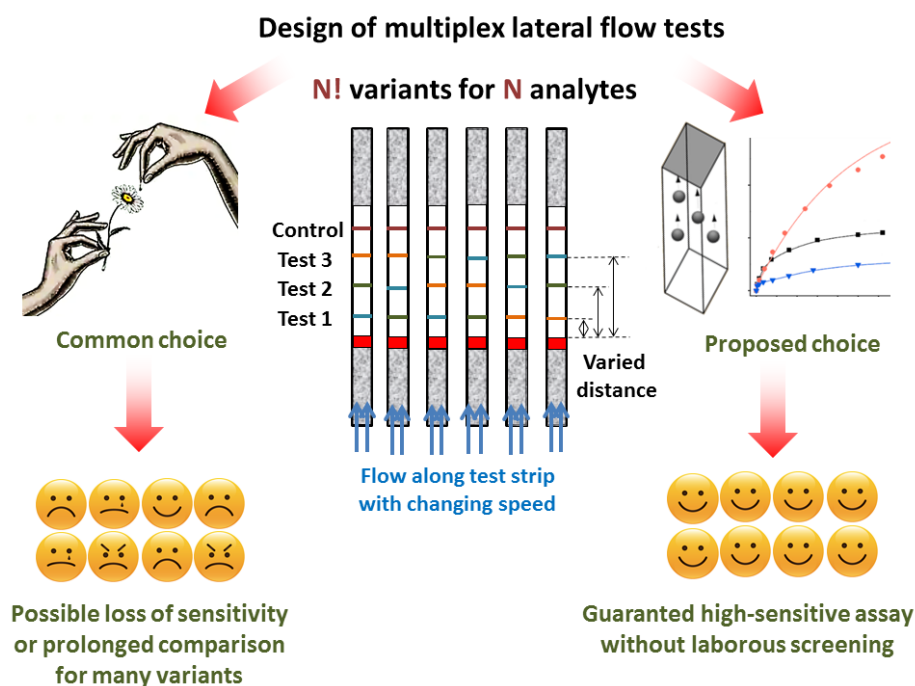
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Abstract: The presented study is focused on the impact of binding zones locations at immunochromatographic test strips into analytical parameters of multiplex lateral flow assay. Due to non-equilibrium conditions for such assays the duration of immune reactions influences significantly on analytical parameters, and the integration of several analytes into one multiplex strip may cause essential decrease of sensitivity. To choose the best location of binding zones, we have tested reactants for immunochromatographic assays of lincomycin, chloramphenicol, and tetracycline. The influence of the distance to the binding zones on the intensity of coloration and limit of detection (LOD) was rather different. Basing on the obtained data, the best order of binding zones was chosen. In comparison with non-optimal location the LODs were 5-10 fold improved. The final assay provides LODs 0.4, 0.4 and 1.0 ng/mL for lincomycin, chloramphenicol, and tetracycline, respectively. The proposed approach can be applied for multiassays of other analytes.

Keywords: Multiparametric assay; rapid tests; immunochromatography; antibiotics; non-equilibrium interactions.

Graphical Abstract

Introduction

The development of analytical techniques for the simple and rapid detection of various compounds is an actual task of high demand. Immunochromatographic assay (ICA) is one of the most efficient approaches for this purpose. It is actively applied for detection of pathogens, biomarkers of infection diseases and functional disorders, hormones and other bioregulators, toxic contaminants of food stuffs and the environments [1-5]. The advantage of ICA lies in the fact that all reactants for its performing are already applied on the test strip before its use. Upon contact of the strip with the tested liquid sample, the liquid removed the applied labeled antibodies, which, in the process of moving along the membrane, specifically bind to the detected analyte in the sample and are fixed on the test zone of the strip. ICA is the simple technique with minimal requirements for sample preparation, and its result is obtained in 5-15 min. The low cost of ICA in comparison with other analytical methods determine their competitive potential as tools for primary mass screening.

Due to increased quantity of controlled compounds in medical diagnostics, food safety and ecological monitoring, there is a need for multiplex test systems. The location of several binding lines with reactants of different specificity is the best technological solution for multiplex ICA [6-9]. Such tests for 2-8 analytes are widely used to determine antibiotics in milk, mycotoxins in grain, etc. and described in a row of recent publications [10,11].

However, the integration of several successfully developed monoplex ICA to one multiplex ICA is associated with several essential problems. The key question in this work is how to store sensitivity of all monoplex ICAs. Combining several reactants in one test strip, we are forced to change the position of the binding zones for different analytes and, accordingly, the conditions of interactions for them. First of all, it is the duration of the interaction in solution, when labeled antibodies and compounds of the sample are moved to the corresponding binding zone. This factor is important, as well as these interactions do not often reach equilibrium, and the degree of its reaching is critical for the number of formed and detected complexes. Also speed of flow movement among the membrane slows down in accordance with the increased distance from the start. This speed essentially affects the sensitivity of the test and the location of test zone in the vicinity of the control zone and at the end of the working membrane is commonly considered as the preferable decision [12]. However, this demand cannot be simultaneously reached for all compounds of multiplex test. Besides, concentrations of interacting reactants are changed as a result of dilution of their initial volumes when the flow moves along the test strip [12,13]. This influences not only to chemical equilibrium in solution, but also to efficiency of labels binding when they reach the test zone. So not only detection limit, but also intensity of coloration (and, respectively, reliability of visual estimation of the assay results) may vary significantly in multiplex tests.

All these factors are stated as important ones in theoretical studies and common guides. However, the published works about multiplex ICA usually do not contain their consideration when choosing a test system design. As a result of this, the transition from monoplex ICAs to multiplex ICA could be associated with losses in coloration of binding zones and a shift in the detection limit. Despite the availability of a number of published developments without indicated with changing the location of the tests zones, the risks of such problems at laboratory level may be considered as significant. So our work was focused on explicit consideration of changes in the assay parameter during the transition to multiplex ICA and finding solution to estimate key parameters of immune reactants and find the best order of their deposition on the test strip. The quantity of the possible orders increases dramatically with the growth of the quantity of test zones (N) and is equal to N! (i.e. 6 for three analytes, 24 for four analytes, 120 for five analytes, etc.). So extensive characterization of all possible variants becomes extremely time-consuming work and should be replaced by simple techniques for assessment of individual reactants. The consideration of triplex ICA seems to be an informative study for such a test of reagents and the identification of their significant characteristics, because explanation of the differences between 6 variants of design in the case of extensive characterization is already becoming very difficult.

Our development for the assay of three antibiotics is important for food safety and medicine, where multitests are especially relevant. Due to the widespread use of antibiotic therapy, bacterial resistance has become the reason for more careful monitoring of their levels in the course of therapy. Tetracycline, chloramphenicol and lincomycin are widely used antibiotics in the treatment of diseases caused by microorganisms. However, improper use of antibiotics has provoked massive resistance to them of many

microorganisms. Such resistance to tetracycline is described for strains of *Pseudomonas aeruginosa*, *Proteus*, *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* [14], to chloramphenicol – for *Bartonella* and *Staphylococcus* [15], to lincomycin – for *Kocuria kristinae*, *Sphingomonas paucimobilis*, *Pantoea*, *Staphylococcus vitulinus*, *Clostridium*, etc. [16,17]. Individual correction of antibiotics dosage decreases the level of antibiotic-resistant bacteria in the intestines [18]. These reasons determine practical importance of multitests for antibiotics not only for food safety control (where successfully commercialized tests are available) but also for planning efficient individual therapy.

Materials and Methods

Reactants

Tetracycline base was from Applichem GmbH (Darmstadt, Deutschland), chloramphenicol base and lincomycin base were from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-tetracycline monoclonal antibody, anti-chloramphenicol and anti-lincomycin (mAb) and hapten-protein conjugates conjugated with bovine serum albumin (tetracycline-BSA (TET-BSA), chloramphenicol-BSA (CAP-BSA), lincomycin-BSA (LIN-BSA)) were purchased in Eximio Biotec (Wuxi, China). Goat anti-mouse polyclonal (anti-species) antibodies were purchased in Arista Biologicals (Allentown, PA, USA). Compounds for preparation and storage of gold nanoparticles - sodium azide, Tween 20, sodium citrate and chloroauric acid – were from Sigma-Aldrich (St. Louis, MO, USA). Pharma Sugar Raffine was from Cristalco (France). Bovine serum albumin (BSA) was from Boval Biosolutions, LLC (Cleburne, Texas, USA). Tris, NaCl, K_2PO_4 and KOH were from Chimmed (Russia).

All the solutions were prepared using deionized water obtained with the use of Milli-Q system (18 MΩ·cm at 25 °C; Simplicity Millipore, Billerica, MA, USA).

Working nitrocellulose membrane CNPC 15 was from MDI (mdi Membrane Technologies, Ambala Cantt, India). Glass-fiber membrane was from Millipore (Billerica, MA, USA), absorbance membrane CF5 was from Whatman (GE Healthcare Bio-Sciences, Marlborough, MA, USA).

Synthesis of gold nanoparticles and their conjugation with antibodies

Gold nanoparticles were synthesized according to standard reproducible protocol for 30 nm nanoparticles as in previous works of laboratory [19]. First, 0.1 mL of 10% $HAuCl_4$ was added to 97.5 mL of filtered 0.18 um Milli-Q water and followed by boiling. After that 1.5 mL of 1% sodium citrate was added and the resulting mixture was boiled for 25 min, cooled and stored at 4°C. Further details of this method can be obtained in the reported study [20].

Anti-mouse IgG antibody (aMAb) dialyzed against 10 mM Tris-HCl buffer (pH 8.6) was diluted by the pre-adjusted to pH of 8.5 colloidal gold by 0.2 M K_2CO_3 solution. For this, 8 mL of colloidal gold was added to 150 uL mAb solution (1000 ug/mL) to the glass flask. The solution was incubated with stirring for 45 min at room temperature. 200 uL of 10% aqueous solution of BSA was added to the mixture with followed incubation under stirring for 15 min at room temperature. The obtained conjugate was separated from unbound antibody by centrifugation at 10,000 g at + 4 ° C for 15 min with decanting of the supernatant liquid. Then 1 ml of 50 mM potassium phosphate buffer, pH 7.4 with 0.1 M NaCl (PBS) containing 0.25% BSA, 0.25% Tween 20, 1% saccharose and 0.05% NaN_3 (TTBSA)was added to the precipitate and used prior to analysis. An optical density of the obtained conjugate was established using a Libra S60 spectrophotometer (Biochrom, Cambridge, UK).

Preparation of the test strips

The LIN-BSA, TET-BSA and CAP-BSA conjugates in a concentration of 1, 0.5 and 0.5 mg/mL respectively and anti-species (GAMI) antibodies in concentration of 0.05 mg/mL from were applied to the nitrocellulose working membrane in an amount of 0.1 μl per 1 mm of the membrane.

After applying the reagents, the membrane was dried for 20 hours at 37° C. Then the membrane was glued together with an absorbing and glass-fiber pad so that in a multimembrane composite the glass-fiber pad was in contact with the sample firstly, and the absorbent - the latest.

The resulting master sheet, using an automatic guillotine cutter, cut the received test strips 3.3 mm wide. Test strips together with a desiccant (0.6 g of silica gel in bags) were sealed in a plastic foil pact and

sealed. Cutting and packaging was carried out at 20–22° C in a special room with a relative humidity of not more than 30%.

Performing lateral flow immunoassay

Samples, working buffer solution and test strips were brought to room temperature (+20-25°C) prior to analysis. Then 5 μL of 20% Tween 20 were added to 100 μL of a sample containing specific antibody against tetracycline and the obtained solution was mixed. A test strip was immersed into the sample for 7 min. Then the test strip was transferred into a working buffer and left for 3 minutes. After this, the test strip was immersed into the working buffer with aMAb gold nanoparticles conjugate having A_{520}=0.5.

Data processing

The dependence of the color intensity from antibiotics concentration in the sample was determined with the Origin 7.5 software (Origin Lab, Northampton, USA). The dependence was approximated using the four-parameter sigmoid function $y = (A − D)/(1 + [x/C]^B) + D$, and the operating range for the quantitative detection of antibiotics was calculated.

Results and Discussion

Location of the test line on the membrane influence analytical parameters even for monoplex immunochromatography [21]. To characterize the corresponding features for all immunoreactants used in our triplex test, we placed the test zone for each analyte in three positions - 4 mm, 11.5 mm and 19 mm. The obtained results for three antibiotics, tetracycline, chloramphenicol, and lincomycin, are given in Figure 1, and comparisons of calibration curves are integrated in Figure 2.
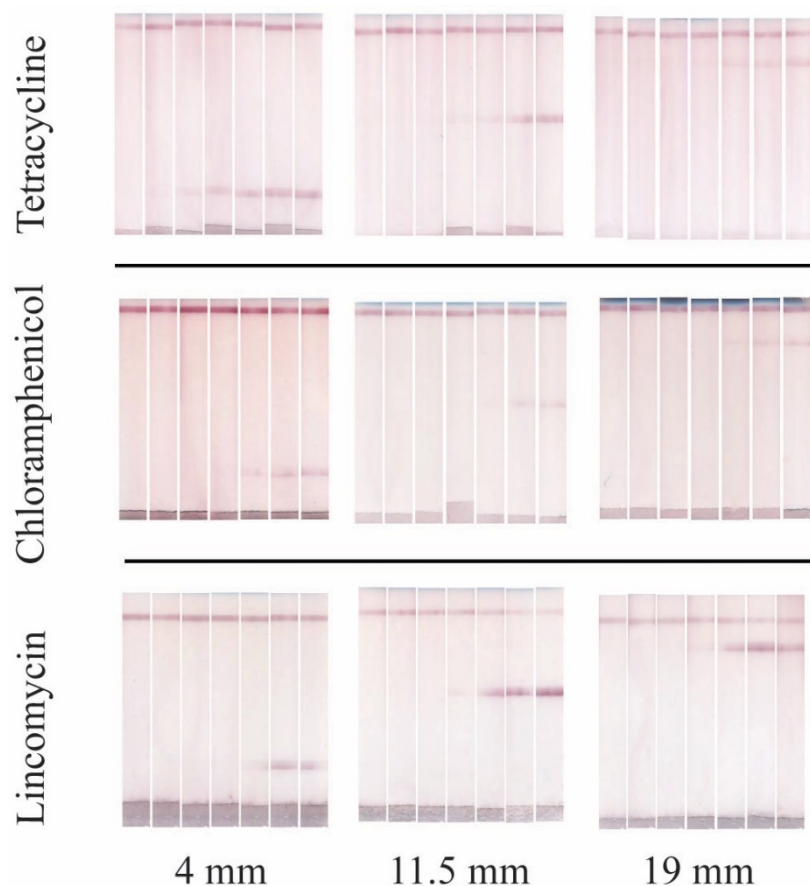

Figure 1. Test strip images after analysis of antibiotics-contained serum samples for different location of test zones. Test strips are arranged from left to right in accordance with the antibiotics concentration (100, 33, 11, 3.7, 1.2, 0.4 and 0 ng/mL)
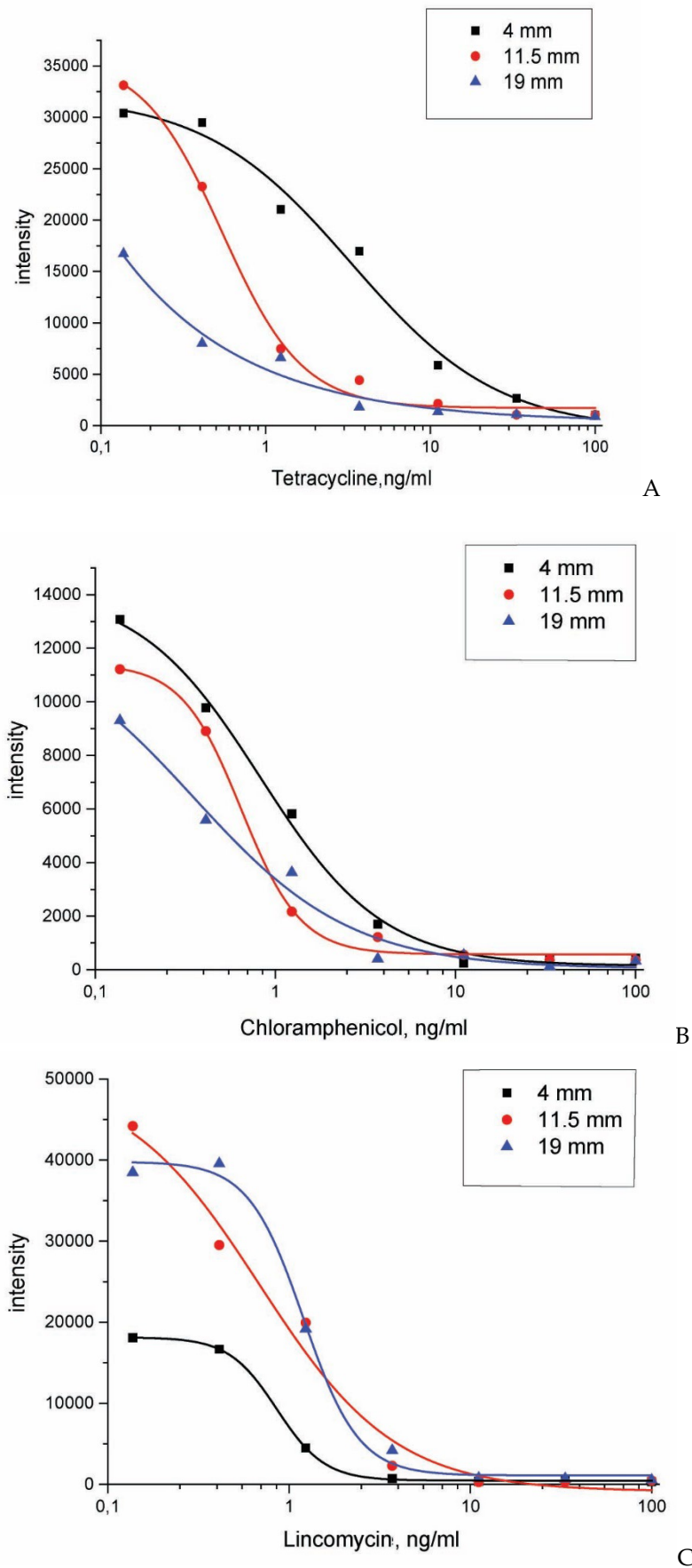
Figure 2. Calibration curves for monoplex lateral flow immunoassays of tetracycline (A), chloramphenicol (B), and lincomycin (C) for different location of test zone.

The obtained results how changes of the intensity of coloration and shift of the working range for each set of immunoreactants. In the case of tetracycline the increased distance of flow leads to better sensitivity, but for 19-mm rum this improvement is associated with significantly decreased coloration. In the case of chloramphenicol changes in location and amplitude of the calibration curves are minimal. The system for lincomicin demonstrates much better sensitivity and appropriate intensity of signal for minimal distance of the test zone from the beginning. Thus, the LOD may be change up to 10 times by simple change of the zone location.

Considering mechanisms causing the demonstrated changes, we should two key factors that affect the flow rate of reagents: (i) size and surface properties of the pores of the working membrane and (ii) the location of the test zone. To estimate these changes, we recorded the flow rate in the course of reactants movement, comparing this parameter for different points along the working membrane. The obtained data are summarized in Figure 3. The reagent flow reached the first test zone (4 mm) at an average rate of 0.83 mm/s. For the second test zone (11.5 mm) the average rate was 0.79 mm/s, and for the third zone (19 mm) it was equal to 0 60 mm/s/
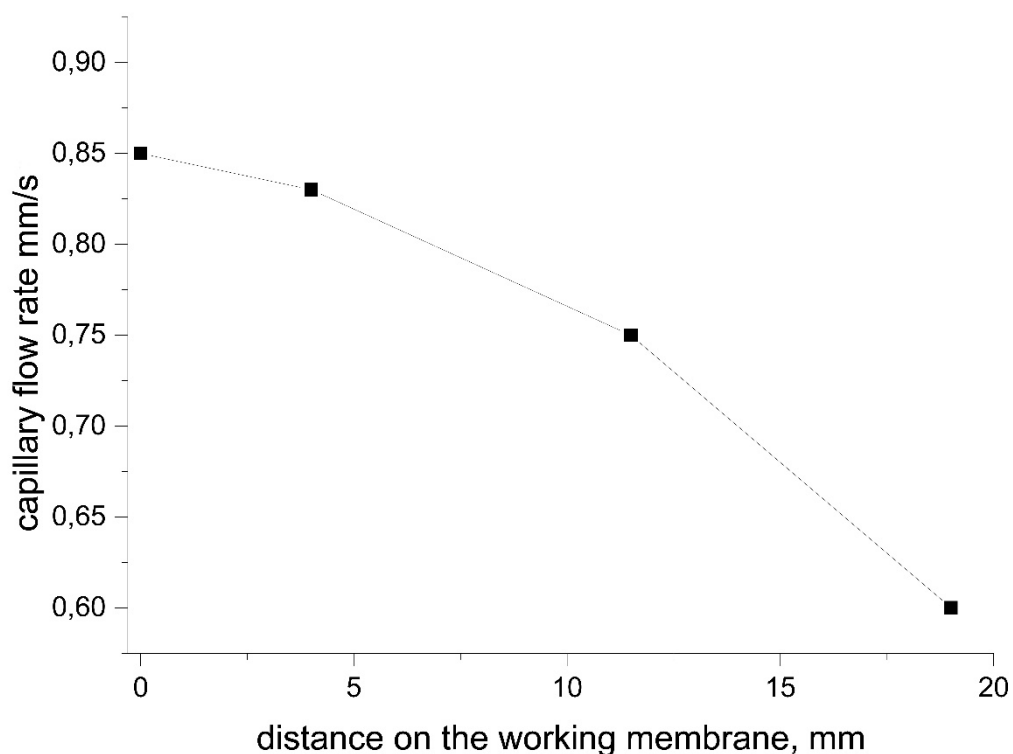

Figure 3. Change in the rate of capillary flow in the course of liquid movement along the working membrane of the test strip.

The exponential decrease of the flow rate in the accordance with the increased distance from the start of the flow was earlier demonstrated for immunochromatographic processes [21.22]. This phenomenon causes the influence of the test zones location on the assay sensitivity. If the line with the applied reagent is located at higher distance from the start, the front of the liquid with the analyte passes more slowly through this line and quantity of the formed bound complexes is increased (more intense coloration). An additional key factor is the time necessary to reach equilibrium for the interactions of reactants in flow. In the case of more affine antibodies the final quantity of immune complexes is formed at the starting part of the working membrane, and additional elongation of the way does not cause improvements in sensitivity. Contrary, for low-affine interactions the choice of maximal distance for the test zone is reasonable. Thus, the preliminary controlled influence of the test tone location on the LOD and intensity of coloration allows separating immunoreactants for multiassay to several groups – with preferable minimal distance of the test zone from the starting point, the preferable maximal distance and without influence of the distance on analytical parameters.

Basing on these regulations and found difference in immunoreactants properties, we have designed a multiplex system where the position of the test zones guarantees a high-quality assay (low LOS and acceptable intensity of coloration). We placed lincomycin at the 4 mm position, chloramphenicol at the 19 mm position and tetracycline at the 19 mm position. The test system demonstrates the instrumental detection limits of 0.4 ng/ml, 0.4 ng/ml and 1.0 ng/ml for lincomycin, chloramphenicol and tetracycline, respectively (see Figure 4 and Table 1).
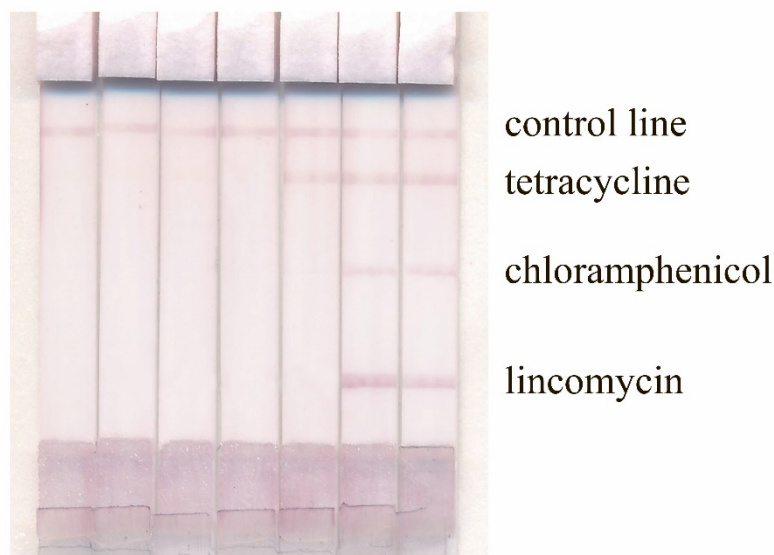

Figure 4. Appearance of the developed multiplex tests for three antibiotics. Test strips are arranged from left to right in accordance with the antibiotics concentration (100, 33, 11, 3.7, 1.2, 0.4 and 0 ng/mL).

Table 1. Analytical parameters (n=3) of the developed multiplex test for three antibiotics

	LOD instrumental, ng/mL	IC_{50}, ng/mL	LOD visual, ng/mL
Lincomycin	0.53 ±0.41	0.83 ±0.73	1.32±0.42
Chloramphenicol	0.46 ±0,32	0.73 ±0.47	1.16 ±0.14
Tetracycline	1.07 ± 0.84	1.70 ± 0.86	2.69 ±0.79

We believe that for some immunoreactants, the location of the test zone at the beginning is critical due to slow immune interactions in solution and limited time of the flow movement along the test zone. The location of the test zone as far as possible from the beginning of the start eliminates these problems. Alternatively, some other reactants provide efficient immune binding both in solution and with immobilized reactants independently on the location of the test zone (see lincomycin example in our study).

Conclusions

In the development of multiplex lateral flow test, we have used data on changes in sensitivity depending on the location of the analytical zones. We showed that only a shift of these zones to the region of lower speeds causes an order of magnitude more sensitive assay. On another hand, for some immunoreactants chemical equilibrium of their interactions is reached rapidly, and so they could be located new the starting region of the working membrane. Using the knowledge about the change in the capillary flow velocity, we constructed a multiplex test for three antibiotics test where the analytical zones are located at a distance that provides the lowest detection limits. Knowledge of these patterns can be applied in the development of various multiplex tests including ones with 4 or more analytical zones and will provide high-sensitive assay for all analytes without laborious screening of all possible variants for immunoreactants location at the working membrane.

Author Contributions

Data curation, Anastasiya V. Bartosh; Formal analysis, Anastasiya V. Bartosh, Dmitriy V. Sotnikov and Anatoly V. Zherdev; Investigation, Anastasiya V. Bartosh and Olga D. Hendrickson; Methodology, Dmitriy V. Sotnikov, Anatoly V. Zherdev and Boris B. Dzantiev; Project administration, Anatoly V. Zherdev; Resources, Olga D. Hendrickson and Boris B. Dzantiev; Software, Dmitriy V. Sotnikov; Supervision, Boris B. Dzantiev; Validation, Dmitriy V. Sotnikov; Visualization, Anatoly V. Zherdev; Writing – original draft, Anastasiya V. Bartosh and Anatoly V. Zherdev; Writing – review & editing, Anastasiya V. Bartosh and Boris B. Dzantiev.

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Conflict of interests

The authors declare that they have no known competition for financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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