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ABSTRACT

The foot-and-mouth disease (FMD) is the most important transboundary viral disease of livestock in the inter-national context, because of its extreme contagiousness, widespread diffusion, and severe impact on animal trade and animal productions. The rapid and on-field detection of the virus responsible for the FMD represents an urgent demand to efficiently control the diffusion of the infection, especially in low resource setting where the FMD is endemic.
Colorimetric lateral flow immunoassay (LFIA) is largely used for the development of rapid tests, due to the extreme simplicity, cost-effectiveness, and on-field operation. In this work, two multiplex LFIA devices were designed for the diagnosis of FMD and the simultaneous identification of major circulating serotypes of the FMD virus. The LFIAs relied on the sandwich-type immunoassay and combined a set of well-characterised monoclonal antibodies (mAb) pairs. One LFIA aimed at detecting and identifying O, A and Asia-1 serotypes, the second device enabled the detection and differentiation of the SAT 1 and SAT 2 serotypes. Both devices also incorporated a broad-specific test line reporting on infection from FMDV, regardless the strain and the serotype involved. Accordingly, five and four reactive zones were arranged in the two devices to achieve a total of six simultaneous analyses. The development of the two multiplex systems highlighted for the first time the relevance of the mAb positioning along the LFIA strip in connection with the use of the same or different mAb as capture and detector ligands. In fact, the excess of detector mAb typically employed for increasing the sensitivity of sandwich immunoassay induced a new type of hook effect when combined with the same ligand used as the capture. This effect strongly impacted assay sensitivity, which could be improved by an intelligent alignment of the mAb pairs along the LFIA strip. The analytical and diagnostic performances of the two LFIAs were studied by testing reference FMDV strains grown in cell cultures and some representative field samples (epithelium homogenates). Almost equivalent sensitivity and specificity to those of a reference Ag-ELISA kit were shown, except for the serotype SAT 2. These simple devices are suitable in endemic regions for in-field diagnosis of FMD accompanied by virus serotyping and, moreover, could be deployed and used for rapid confirmation of secondary outbreaks after FMD incursions in free-areas, thus contributing to promptly implement control measures.

1. Introduction

Foot-and-mouth disease (FMD) is an extremely contagious viral disease of livestock [1], which affects cattle, swine, sheep, goats and other cloven-hoofed ruminants, including many wild species. Due to its severe impact on animal trade and animal productions, it is the most important transboundary animal disease in the international context. It causes annual costs due to production losses and vaccination estimated at €5.3–€17 billion (US$6.5–US$21 billion) in endemic areas [2,3]. FMD is widespread throughout the world, particularly in Asia, Africa and the Middle East [3,4], and infected countries pose a permanent threat to free-countries due to risks of introduction, exacerbated by globalization. Improving disease control at source represents, therefore, a mutual in-terest for both endemic and free-countries, and this led OIE/FAO to endorse a Global FMD Control Strategy [5].

The disease causative agent, FMD virus (FMDV), belongs to the genus Aphtovirus, family Picornaviridae; it exists as seven antigenically distinct serotypes (O, A, C, Asia1, and Southern African Territory (SAT 1, 2 and [3,6]) and evolutionary pressure leads to continuous emergence of new variants within each serotype which tends to remain confined into distinctive geographical areas. When an outbreak is suspected based on clinical presentation, confirmation of FMD by means of diagnostic techniques is essential for the implementation of effective measures to contain the spread of the disease. Confirmatory laboratory diagnosis relies on virus detection in clinical samples, that can be achieved using a panel of different techniques, of which the most common include Virus Isolation (VI), enzyme-linked immunosorbent assay (ELISA) systems or a variety of nucleic acid detection methods [7]. Despite their reliability and accuracy these diagnostic tests are labour intensive and need to be operated in equipped laboratory setting by trained personnel. As an alternative, "pen side tests" exploiting the lateral flow immunoassay (LFIA) technique, have been developed for the rapid and on-field detection of the virus.

The LFIA platform is largely used for the development of point-of-care testing (POCT), as it guarantees rapid results (less than 20 min), extreme simplicity, cost-effectiveness, and on-field operation [8–10]. Typical LFIAs for POCT application are qualitative assays, with colori-metric detection enabling the visual inspection of results. Gold nano-particles (GNP) or dye-encapsulated microspheres are employed as signal reporters, and are linked to specific recognition elements, such as antibodies. Diagnosis of viral infections for example can be obtained through the detection of viral antigens, which are captured by specific antibodies coated onto the porous membrane. The simultaneous binding of the viral antigen to the coated and detection antibodies results in the accumulation of the signal reporters in a spatially confined zone indi-cated as the "test line", and, ultimately,

to the colouring of the line. As several reactive zones can be created by coating different antibodies in separate lines, the LFIA is also suited for multiplexing immunoassay.

LFIAs for the detection of FMD virus based on the sandwich-type assay with pair of specific antibodies have been described [11] and Table 1 summarizes their main characteristics. Some authors used inter-types cross-reactive antibodies (usually indicated as “PAN-FMDV”) for setting up devices capable of diagnosing the FMD, without differ-entiating on the virus serotype [12,13]. These devices detected any strain of FMDV serotypes O, A, C and Asia 1, with one of them reporting moderate reactivity with also the SAT serotypes. Type-specific LFIAs for detection of single serotypes have been described as well [11,14–21], though most of them were evaluated on limited numbers of experi-mental samples or field samples representative for topotypes present in a restricted geographic region. In most countries where FMD is endemic, multiple FMD virus serotypes co-circulate, and identification of the specific serotype involved in outbreaks is vital to ensure that vaccines are appropriately tailored to protect the animals. These endemic regions often lack adequate transport systems, equipped laboratories, and the expertise to provide a centralized testing service.

Multiplexing LFIA enabling the rapid and easy detection with simultaneous serotyping of FMDV in clinical samples is therefore a best option in such situations, as LFIAs can be deployed on infected farms as pen-side tests or in mobile laboratories. The feasibility of multiplexing LFIAs, including several test lines with different specificity has been explored. In particular, Yang et al. developed a single device including three test lines formed by FMDV O-, Asia 1-, and A-type specific anti-bodies to capture selectively the corresponding serotype, and a cross-reactive labelled antibody to stain captured antigens [17]. In this assay the type-specific antibodies had to be labelled with a different marker for each serotype to be captured on commercial strips providing three marker-mediated capturing systems. The multiplexing capability was further increased in the work of Morioka et al. who added a C-type specific and a PAN-FMDV test line enabling the detection of the seven FMD serotypes by the PAN-FMDV line and the simultaneous differenti-ating of up to four serotypes (O, A, Asia 1, and C) by the corresponding type-specific lines [16]. The evaluation of this device, however, was only focused on testing of field samples from a single epidemic (Japan 2010) caused by a type O FMD virus, in addition to some experimental samples harbouring the same type O strain or one type A strain. Multiplexing typing of the SAT serotypes remains unexplored.

More recently, the lateral flow assay technology has been exploited for revealing the product of the recombinase polymerase amplification (RPA-LFD) as diagnostic tools for FMD diagnosis [22,23]. In these works, the RPA-LFD devices were able to detect separately the viral RNA of O, A, and Asia 1 FMDV types with sensitivity comparable to the one of bench-top PCR.

With the aim of establishing an ultimate LFIA device capable of detecting and differentiating FMDV serotypes, a set of two multiplexing lateral flow devices (LFDs) for the diagnosis of FMD and the simulta-neous identification of major circulating serotypes of the FMD virus was established based on sandwich-type colorimetric immunoassays. We used five mAbs combinations specific for O, A, Asia 1, SAT 1 and SAT 2 serotypes divided in two LFDs according to the geographical distribution of FMDV serotypes [3,4]: the so-called “Eurasia LFD” enables detection and identification of FMDV O, A and Asia 1, while the “Africa LFD” is addressed at detection and differentiation of the SAT 1 and SAT 2 se-rotypes. In addition, the PAN-FMDV test was included to complement the specific typing and to detect additional serotypes (C or SAT 3) and possibly new, or mutated, FMDV variants which might escape binding to the selected serotype-specific mAbs. Reference FMDV strains grown in cell cultures were used to develop and investigate the performances of the new diagnostic devices, in comparison with ELISA tests. Moreover, an initial evaluation of the two LFD prototypes was conducted to verify matrix effect and on-field feasibility, using some representative field samples (epithelium homogenates) including four of the five FMDV serotypes.

Interestingly, during LFD development, we encountered for the first time an “uncommon” hook effect, which occurs when sandwich assays are realized in the lateral flow immunoassay platform using the same antibodies as capture ligand and detector. Therefore, we investigated the unusual hook effect and proposed a model for interpreting it. Ac-cording to the model, we also suggested a general route for designing multiplexing LFIA in which antibodies are interconnected (because of the possible competition between intertypes-reactive and type-specific mAb pairs in the same reaction or because the same bioligand is used for capturing and detection).

2. Materials and methods

2.1. Immunoreagents, chemicals and materials

Gold (III) chloride trihydrate (ACS reagent), anti-mouse immuno-globulin G antibody produced in goat, casein sodium salt from milk, sucrose, and bovine serum albumin (BSA) were obtained from Sigma-

–Aldrich (St. Louis, MO, USA). Tween20 and other chemicals were purchased from VWR International (Milan, Italy). Nitrocellulose mem-branes (CNPC-SS12) with cellulose adsorbent pad and glass fibre BR4 sample pads were purchased by MDI membrane technologies (Ambala, India). Glass fibre conjugate pads were obtained from Merck Millipore (Billerica, MA, USA). Statistical calculations were carried out with Sig-maPlot 11.0 software.

Six anti-FMDV type-specific mAbs (#2H6, #3D8, #3B11, #2A10, #5F6, and #4D12) and the PAN-FMDV mAb (#1F10) used for gold nanoparticles conjugates and to form the test lines were generated at the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, while the hybridoma for the production of the anti-FMDV SAT1 mAb #HD7 was generated at The Pirbright Institute, UK. Details on the mAbs, including their specificity and references, are displayed in Table S1.

The Extraction kit for tissue homogenization was provided by In3Diagnostics srl (Torino, Italy). The medium used for the extraction was also used to dilute inactivated supernatants of FMDV infected cell culture and epithelium homogenates.

2.2. Preparation of the colorimetric probes: labelling anti-FMDV mAbs with GNP

GNPs with a localized surface plasmon resonance (LSPR) band cen-tred at 525 nm and mean diameter of ca. 30 nm were prepared by gold

(III) chloride trihydrate reduction with sodium citrate [24]. Signal re-porters used in the LFIA devices were prepared by adsorbing the #1F10 (PAN-FMDV), #2H6 (anti-FMDV SAT 2), and #HD7 (anti-FMDV SAT 1) mAbs onto GNPs, separately. The optimal antibody/GNP ratio for conjugation was determined by the flocculation stress test [25,26]. Briefly, concentrated sodium chloride was added as the aggregation promoter, to GNP-antibody conjugates obtained from variable GNP/Ab ratios. When the GNP-antibody conjugate is shielded by the mAb, no salt-induced aggregation occurs. According to the stress test, the optimal amounts for adsorbing mAbs to 1 ml of GNP at optical density (OD) equal to 1 were found as 4 μg, 6 μg and 6 μg of #1F10, #2H6, and #HD7 mAb, respectively (details on the flocculation stress test are reported in the ESI; results are shown in Fig. S1).

For detectors preparation, the appropriate amount of the mAb was added to 10 mL of pH adjusted GNP (pH 8.5). The solution was gently stirred for 30 min at 37 ◦C. Then, 1 mL of 1% (w/v) BSA in borate buffer (20 mM, pH 8) was added and the mixture was incubated for 10 min at 37 ◦C. Finally, the GNP–mAb conjugate was recovered by centrifugation (10 min at 7100×g), washed once with borate buffer supplemented with 3

0.1% BSA, and reconstituted in the same buffer supplemented with 2% (w/v) sucrose and 0.25% (v/v) Tween 20. GNP-mAb probes were stored at 4 ◦C until use.

2.3. Optimization of the Eurasia and Africa LFIA devices

Parameters studied for the development of the devices included: (i) the concentration of the coated mAbs; (ii) the amount of the probe; and

(iii) the material of the sample pad. MAbs were coated at 0.5 and 1 mg/ ml. Based on previous knowledge on mAbs reactivity [27,28], the optimal capture of type O and type A FMD viruses required a combi-nation of two mAbs for each serotype. The same ratios optimized for the FMDV antigen detection and serotyping ELISA kit were employed for the LFD, namely 1 + 1 (#3B11 + #2A10) for O-type and 0.75 + 1 (#5F6 + #4D12) for A-type. In these cases, the concentration of the coated re-agent was considered as the individual mAb concentration (i.e. 1 mg/ml 3B11 + 1 mg/ml 2A10 were mixed to form the "1 mg/ml" line). The optimal amount of the probe included in the LFD for the Eurasia device (#1F10-GNP) was checked by diluting the #1F10-GNP conjugate to OD 0.5, 1 or 2. Since the LFD probe for the Africa device was made by a combination of three mAbs (#1F10, #2H6, and #HD7), the antibodies were separately adsorbed onto GNP and then mixed in variable ratios. The mAbs #2H6 (anti-SAT 2) and#HD7 (anti-SAT 1) were mixed 1 + 2; 1 + 1; 2 + 1. Once defined their optimal proportion, the #1F10-GNP was added. Samples pads from different manufacturers were compared (GF-BR4 by MDI, Ambala, IN and GF/DVA by Whatman, Maidstone, UK). Criteria used to judge the results were: no signal appearing at test lines for the negative control antigen (and for heterologous FMDV types) and the more intense colour observed at each line for the specific ho-mologous FMDV type. To compare colour intensity, images of the strips were acquired by a benchtop scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and processed by QuantiS-can 3.0 software (Biosoft, Cambridge, UK).

2.4. Investigation of the 'hook effects'

To shed light on the empirical observation that the sensitivity of the PAN-FMDV test line in the Eurasia LFD depended on its positioning with respect to the sample application point, we measured the colour of the PAN- and type-specific test lines as a function of the amount of the de-tector (anti-PAN-FMDV antibody labelled with GNP), the amount of the antigen (inactivated suspensions of type O- A- and Asia 1-FMDV refer-ence strains grown in cell cultures), and distance from sample applica-tion point. In details, we applied serially diluted inactivated suspensions of FMDV to the Eurasia device, in which the PAN-FMDV line was the first starting from sample well, and in which the probe was used at OD 2. For studying the effect of the probe amount, we fabricated new devices, in which the probe was applied at OD 3 and 4. The inactivated suspensions of FMDV were analysed at 1:10 dilution. Finally, to investigate the effect of the line positioning, the experiments were repeated on a new device, which was fabricated by drawing the PAN-FMDV line as the fourth line (last test line of the multiplex configuration).

Similarly, the investigation was repeated for the Africa LFD, using SAT 1 and SAT 2 reference isolates and measuring colour of the test lines as a function of the amount of the detector (#2H6-GNP or #HD7-GNP, respectively), and of the amount of the antigen. Also in this study, the antigens were varied by serially diluting the inactivated suspension of the virus, while the amount of the two probes was studied by ad-hoc fabricating LF devices in which the GNP-mAb was applied at OD 3 and 4.

2.5. Production of the Eurasia and Africa LFIA devices

The various capture antibodies used for drawing test and control lines of the LFIA devices were diluted in phosphate buffer (20 mM pH 7.4) at the final concentration of 1 mg/ml and applied at 1 μL/cm onto the nitrocellulose membrane by means of a XYZ3050 platform (Biodot, Irvine, CA, USA), equipped with BioJetQuanti™ 3000 Line Dispenser for non-contact dispensing. The order of the test lines allowing for optimal analytical performances is depicted in Fig. 1. The LFD Eurasia device included 5 lines, in the following order: the PAN-reactive mAb (#1F10); a 1:1 mixture of two O-type specific mAbs (#3B11 and #2A10); the Asia 1-specific mAb (#3D8); a mixture of two A-type reactive mAbs (#5F6 and #4D12) in the ratio 0.75:1 and the anti-mouse immunoglobulin as the control line. Lines were drawn at 3 mm distance each other. The probe for the LFD Eurasia was composed by the unique PAN-reactive mAb #1F10-GNP (OD = 2). The Africa device was designed as a 4-lines LFIA strip where the first line contained the SAT 1-specific mAb (#HD7) and the second the SAT 2-specific mAb (#2H6). The third line contained the PAN-FMDV reactive mAb (#1F10) and the fourth was set for the control line. As the signal reporter, we used a mixture of #1F10-GNP, #HD7-GNP, and #2H6 in the ratio 1:2:2 (total OD = 5).

The gold probes were diluted to optimal OD with GNP dilution buffer (borate buffer with 0.25% Tween 20, 2% sucrose and 0.02% sodium azide), adsorbed onto a pre-saturated glass fibre conjugate pad and dried for 4 h at room temperature. Strips were composed as follows: sample pad, conjugate pad, membrane, and adsorbent pad and were cut in 4.2 mm-width by means of a CM4000 guillotine (Biodot, Irvine, CA, USA). Finally, strips were inserted into plastic cassettes (Eximio Biotech, China) to fabricate the ready-to-use LFIA device. Cassettes were stored in the dark in plastic bags containing silica at room temperature until use.

2.6. Viruses and samples

Inactivated supernatants of FMDV infected cell cultures were used for the optimization and evaluation of LFIA. Reference FMDV strains of serotypes O, A, C, Asia1, SAT 1 and SAT 2 were propagated in IBRS-2 cells monolayers and harvested when cytopathic effect (CPE) was maximum. After clarification by centrifugation followed by filtration through 0.2 μm filters, the FMD virus was inactivated with binary eth-yleneimine. In particular, the following reference strains for the six se-rotypes were used for tests optimization: FMDV O Manisa, A22 Iraq, C1 Brescia 64, Asia 1 Nepal 29/97, SAT 1 Botswana 1/68 and SAT 2 Zimbabwe 5/81. In addition, an inactivated suspension of swine vesic-ular disease virus (SVDV), strain Italy '72, was used as a negative anti-gen. Two or three FMDV strains for each serotype, in the format of infectious culture supernatants, were used for analytical sensitivity studies: for each virus suspension the concentration of infectious virus was determined by titration and expressed as TCID50/ml (50% tissue culture infective dose).

For matrix effect experiments and for initial evaluation of the new devices, the tongue of a healthy cow was collected at slaughterhouse following routine dissection. Tongue epithelium homogenization was made according with the protocol of the extraction kit (In3Diagnostics srl, Torino, Italy), in compliance with OIE Manual of Diagnostic tests and vaccines for terrestrial animals [29]. An amount of ca 200 mg of epithelium was cut and added with 1 ml of the diluent buffer. After an extensive grinding of the mixture, the solid residue was decanted, and approximately 100 μL of the supernatant were dispensed in the sample well of the device. Furthermore, vesicular epithelium homogenates from 21 FMD outbreaks occurred in Tanzania during 2012–2018 were ana-lysed using the multiplex LFIA; the samples were confirmed positive for one of the different FMDV serotypes O, A, SAT 1 or SAT 2 by VI tests and topotype-specific real-time RT-PCR [30,31]; results of LFIA were compared with those provided by an FMDV Antigen detection and serotyping ELISA kit, based on the same mAbs used to design the multiplex LFIA [27,28].

For testing by the LFIA, the supernatant of virus cultures and epithelium homogenates were diluted 1 + 1 with the diluent buffer and applied to the sample well of the cassette.
The result was visually inspected after 20 min from sample application.

2.7. Analytical evaluation of the Eurasia and Africa LFDs

Selectivity of the devices was evaluated by analysing reciprocal cross-reactivity of the various FMDV types on heterologous test lines. Also, FMDV of types O, A, and Asia1 FMDV were applied to Africa LFD, and FMDV SAT 1 and SAT 2 were applied to the Eurasia LFD.

Matrix effect was studied by analysing tongue extracts from a healthy cow before and after spiking with FMDV of the various serotypes.
The analytical sensitivity was studied by testing serial dilutions of infectious cultures of FMD viruses (one or two different topotypes per each serotype), with a known infectious titre (expressed as TCID50/ml). The limit of detection was defined as the sample dilution that showed a perceivable colour at the test line, as judged coherently by three oper-ators. The sample dilutions were measured in parallel by means of the reference Antigen-ELISA kit.

2.8. Antigen-ELISA kit (Ag-ELISA)

Detection and serotyping of FMDV by Ag-ELISA was carried out using an updated version of the FMDV Antigen Detection and Serotyping ELISA Kit (IZSLER, Brescia, Italy and TPI, Pirbright, UK) [27], including detection and typing of serotypes SAT 1 and SAT 2, in addition to O, A, C and Asia 1. Manufacturers' instructions were followed. Briefly, original tissue homogenates or cell culture supernatants were diluted 1:2 and incubated for 1 h at room temperature into one row of the ELISA plate, precoated with the battery of selected type-specific capture mAbs and one additional pan-FMDV mAb. The same pan-FMDV mAb conjugated to peroxidase was used to complete the detection and typing of serotypes O, A, C and Asia 1, while a conjugate comprising one SAT 1, one SAT 2 and one cross-reactive mAb was used for the detection and typing of SAT 1 and SAT 2 serotypes. The two conjugates were incubated for 1 h at

room temperature, after which time the reaction was developed using TMB (3,3′,5,5′-tetramethylbenzidine) as substrate and stopped after 20 min with addition of H_2SO_4 0.6 N. Optical density was measured at 450 nm using an ELISA reader. Fifty µL/well of each component were delivered for each incubation step and washes between steps were performed.

3. Results and discussion

3.1. Development of the Eurasia and Africa LFIA devices

The mAbs used for the development of the two LFDs (Table S1) as well as their combinations as capture and GNP-conjugated mAbs were previously described and used in validated ELISA assays for FMDV detection and typing [28]. Selection criteria for the type-specific mAbs included type-selectivity associated with broad intra-typic reactivity, to allow detection of old and emerging antigenic variants. A mAb cross-reacting with all serotypes was used to create an additional PAN-FMDV test line, for the detection of new variants which might escape binding to the selected serotype specific MAbs.

3.2. Eurasia LFD

The Eurasia LFD was developed first and was designed to allow for the simultaneous typing of the three FMDV serotypes O, A and Asia 1; it was complemented with an additional pan-FMDV detection to recognize new variants which might escape binding to the selected serotype-specific mAbs. Therefore, four test lines were drawn, as shown in Fig. 1 (a, b, e). The detection sites were formed by coating the PAN-FMDV mAb (#1F10), a 1:1 mix of the two O-specific mAbs (#2B11 and #2A10), the Asia 1-specific mAb (#3D8), and a 1:0.75 mix of the two A-specific mAbs (#4D12 and #5B6) to form the PAN-FMDV and the three type-specific lines, respectively. The proportion of mixed mAbs for the serotypes O and A were defined according to their reactivity previ- ously measured for the Ag-ELISA kit. The probe was formed by the GNP-labelled mAb #1F10, i.e., the inter-types cross-reactive mAb (PAN-FMDV).

Preliminary experiments allowed us to conclude that a final con- centration of 1 mg/ml of coated antibodies in each line was sufficient to assure intense colouring of the type-specific lines in the presence of the homologous FMDV samples (corresponding to 1/10 dilution in extrac- tion buffer of inactivated supernatants from infected cell cultures). Lower amounts decreased the signal, while higher amounts did not produce an appreciable increase of the colour at the lines (data not shown). Similarly, increasing the optical density of the #1F10-GNP probe from 1 to 2 was shown to increase the colour at the type-specific test lines, while a further increase resulted in a higher background without significantly improving the detectability (data not shown).

As far as the PAN-FMDV line, we initially opted for positioning the PAN-FMDV mAb as the farthest from sample application, so that type-specific interactions were favoured (Fig. 1a). However, the sensitivity of the PAN-FMDV line was very poor, whatever FMDV type was used as the sample (Fig. S2). The hypothesis was made that the capture of the antigens by the type-specific lines prevented them to reaching the PAN-FMDV line. To increase the response at the cross-reacting line, the PAN-FMDV antibody was then used to create the first reactive zone encountered by the sample during the flow (Fig. 1b). This configuration enabled recovering the sensitivity of the PAN-FMDV without interfering significantly with the detection capability of the type-specific lines (Fig. S2).

3.3. Africa LFD

The Africa LFD included two specific test lines formed by the anti-SAT 1 (#HD7) and anti-SAT 2 (#2H6) mAbs. Typing of serotype SAT 3 was not included due to the lack of specific pair of mAb for this serotype, which, anyway, is the least occurring and confined to a restricted area in southern Africa. Despite the incomplete cross-reactivity of the PAN-FMDV mAb #1F10 with SATs serotypes, a so-called PAN-FMDV test line was included in the Africa device as well, considering that the #1F10 mAb is able to recognize many SAT 1 and part of SAT 2 FMD viruses and that a multiplicity of FMDV O and A topotypes also circulate in African countries. The same mAbs were also labelled with GNP and mixed with the PAN-FMDV probe 1F10-GNP to allow typing of SAT 1 and SAT 2 FMD viruses in the type-specific lines and detecting of the majority of FMD viruses irrespective the serotype in the PAN-FMDV test line. The optimal proportion and the absolute amount of each type-specific probe were investigated by maximizing colouring of both type-specific lines while limiting background signal. According to the quantitative analysis of images, the following condi- tions were established: mixing the probes in equal proportion and reaching a final concentration of each mAb-GNP corresponding to OD2. The PAN-FMDV 1F10-GNP probe was then added to the mix of anti-SATs-GNP. Based on the previous observations with the Eurasia LFD, the PAN-FMDV line was positioned near to the sample well followed by the SAT 1 and SAT 2 specific lines, respectively (Fig. 1c). However, the insertion of the PAN-FMDV line caused a dramatic loss of sensitivity in the type-specific lines, mainly for the SAT 1 detection. Coherently with the cross-reactivity of the PAN-FMDV mAb with the SAT 1 strain, most of the SAT 1 antigen was apparently subtracted by the PAN-FMDV line and did not reach the type-specific line. Therefore, the ultimate arrangement of reactive sites was established for the Africa LFD as follows: SAT 1 and SAT 2 test lines first, followed by the PAN-FMDV one, finally ending with the control line (Fig. 1d). The probe was formed by a mixture of three mAb-GNP conjugates (i.e.: 1F10-, HD7-and 2H6-GNP in the optimal proportion of 1:2:2).

3.4. Evidence of a new type of the hook effect

The empirical observation on the significant effect due to the use of two reaction lines for the same antigen, of which one cross-binding to any FMDV antigens irrespective the serotype, led to further investigate on the intelligent arrangement of reactive zones. In fact, the two devices had opposite optimal configurations: the alignment of capture mAbs included the PAN-FMDV as the first and as the last bioligand in the Eurasia and Africa LFDs, respectively. Of course, the different relative affinities of the various mAbs and the exposure of their target epitopes may explain the

observed behaviour. Nevertheless, we noticed also that when O-, Asia 1-, and A-FMD viruses were applied to the Africa LFD (which included only one reactive site for these serotypes, i.e., the PAN-FMDV site), the signal was significantly lower compared to the one provided by the same samples when applied to the Eurasia LFD, despite the PAN-FMDV detection included the same capturing and detector mAb in both configurations. The net effect of distancing a reactive site from the sample well is the increase of the contact time between the sample and the detector before they reach the capture site. We supposed that the interaction between the viral antigens and the detector antibody, which occurs in solution during their flowing across the membrane, could led to the saturation of the antigen epitopes thus preventing the subsequent binding to the capture antibody. The suggested effect should impact on sandwich assays based on the same capture and detector mAb, which bind simultaneously to equivalent epitopes on the same viral particle. In fact, we observed that the positioning of the reactive line using the same mAb for capture and detection was the most critical, either for the PAN-FMDV or for the SATs-specific antibody pairs in the Eurasia and Africa LFDs, respectively. To shed light into these findings, we measured the colour formed at the several test lines as a function of the quantity of the detector and of the antigen.

Starting from the Eurasia system, we found an almost linear rela- tionship between the colouring of lines (including the control line) and the amount of the detector (Fig. S3), except for the PAN-FMDV line (Fig. 2). The behaviour was qualitatively similar whatever FMDV sero- type was used as the sample. In the case of the PAN-FMDV sandwich (same capture and detector antibody), initially the increase of the de- tector amount provoked a parallel increase of the signal, followed by a saturation or a decrease of the signal, which is typical of the so-called “hook effect” [32]. However, the hook effect is correlated to an excess of the antigen rather than excess of the antibodies used for its detection. In the classic hook effect, when the quantity of the antigen surpasses the linearity range, the probability of the simultaneous binding of the cap- ture and detector antibodies to the same antigen molecule or viral particle to form a three-terms complex (capture antibody-antigen-detection antibody) decreases. In place of the three-terms complex, two two-terms complexes form, such as the cap- ture antibody-antigen and the antigen-detection antibody ones. There- fore, the excess of the antigen prevents or reduces accumulation of the detector at the test line (Fig. 2b). However, we did not observe a decreasing colour while increasing antigen amount. Conversely, in the present work, we observed a saturation effect when increasing the amount of the detector. The excess of the probe saturates the antigen reducing availability of epitopes for binding to the capturing antibody (Fig. 2a). The order of the interactions occurring in flow (the antigen comes into contact with the detector first, and then to the capture antibody) corroborated the suggested inhibitory mechanism. To support our hypothesis, we carried out two further experiments: in one case, we varied the amount of the antigen and, in a second one, we repeated the same study as above described but shifting the pan-FMDV test line (incorporating the same mAb as capture and detector) so that the con- tact time between the antigen and the probe before reaching the test line was increased. As shown in Fig. S4, a qualitatively superimposable relationship between the antigen quantity and the signal measured was found at both the type-specific and PAN-FMDV lines, independently on the FMDV serotype tested. This result indicated that the signal dependency on antigen amount was similar for both the sandwich including one mAb or two different mAbs for the capture and detection of the antigen. On the contrary, and coherently with the hypothesis made, the shift of the PAN-FMDV line farthermost resulted in the exasperation of the hook effect, resulting in a general loss of sensitivity compared to the system with the PAN-FMDV line near to the sample well. In addition, the higher the amount of the probe, the more pro- nounced the hook effect (Fig. 2c), which strengthened the suggestion of antigen saturation by the detector antibody.

The conclusions drawn by the study were confirmed and reinforced when considering the Africa device. In a simplified version of this as-sembly, not including the PAN-specific antibody as a detector, the SAT 1 and SAT 2 type-specific reactions were obtained using the same capture and detector mAb for the relevant sandwich assays. Again, we observed the atypical hook effect (Fig. S5) for the two type-specific sandwich assays (same mAb for capture and detection), while the PAN-FMDV line (in which the capture antibody and the detector were different) pro-vided a linear ‘signal vs probe amount’ relationship. Therefore, we concluded that the inhibition of antigen binding to the capture antibody, due to the saturating effect of the detector including the same mAb could be generalized. Although, other authors have already evidenced that the amount of the probe should be optimized in order to reach maximum sensitivity in sandwich LFIA [33], we shed light in the mechanism of the decreased sensitivity and showed that the position of the capturing re-agent is a key-point to reduce this adverse effect, as the maximum signal achievable by a “distant” reactive zone remained lower than the one provided by test lines near to the sample well. These findings also implied that the antigen-probe interaction was extremely rapid. Indeed, the few seconds elapsed between probe resuspension within the sample and their reaching the “near” reactive zone were sufficient to form the complex.

3.5. Preliminary evaluation of the LFDs diagnostic performance

Analytical specificity and sensitivity were evaluated using inactivated cell culture supernatants of each FMDV serotype as reference antigens. Specificity estimates were derived by checking the cross-testing of each FMDV serotype on the other type-specific lines in both Eurasia and Africa devices: all samples were negative in heterologous tests, while type-specific and PAN-FMDV lines reacted consistently with the sero- type analysed. In addition, the specificity was further evaluated by testing infected culture supernatants of SVDV, another picornavirus causing lesions in pigs indistinguishable from FMD, with negative results (Fig. 3).

The analytical sensitivity of the two LFDs was evaluated by testing serial dilutions of two-three reference FMDV strains for each of the five serotypes (O, A, Asia 1, SAT 1, SAT 2) with known infectious titres (expressed as TCID50/ml). The Ag-ELISA kit was run in parallel as the best matching laboratory test, considering that it is based on the same mAbs sandwich configurations to reveal the antigen-antibody reaction.

The Eurasia LFD showed equivalent analytical sensitivity for the detection of FMDV serotypes O and A, corresponding to a detection limit of 10^4 TCID50/ml (approximately 10^3/test), while sensitivity was slightly lower for the serotype Asia 1, with a detection limit ranging between 10^4 and 10^5 TCID50/ml (10^3-10^4/test). Analogous detection limits were observed with both type-specific and PAN-FMDV lines, and these were overlapping with results provided by the Ag-ELISA kit.

With the Africa LFD, a viral load of 10^4-10^5 TCID50/ml (10^3-10^4/test) was necessary to obtain a positive signal for FMDV SAT 1 and at least 10^5 TCID50/ml (10^4 /test) for SAT 2. For these two serotypes the Ag-ELISA kit exhibited an analytical sensitivity approximately 4-fold higher.

However, it is also known that in field samples, due to inadequate storage conditions, the virus can be degraded or have partially lost infectivity, while maintaining the antigenic concentration and reac-tivity. To verify this condition, four archived field samples (vesicular epithelium homogenates), positive for each of FMDV serotype O, A, SAT 1 and SAT 2 respectively, were also submitted to virus titration and tested in serial dilutions with the LFDs and the Ag-ELISA kit. For all four samples the apparent analytical sensitivity improved by 100–1000 orders of magnitude compared to that evaluated using viruses recently refreshed by cell culture passage, thus confirming that virus degradation frequently observed in field samples does not affect the capability of the LFDs to detect the FMD viral antigens, in contrast to VI.

To evaluate the matrix effect, due to the possible interference of co-extracted substances, the epithelium from the tongue of a healthy cow was weighted, roughly minced, and then extracted according to the protocol of the extraction kit. The procedure was repeated on three slices of the sample and the extracts were directly analysed by the LFDs. Among the investigated materials for the sample pad, the glass fibre GF-BR4 was selected as the one which showed no signal for any test lines. Then, extracts were fortified by adding separately O-, A-, and Asia 1-type inactivated cell culture supernatants at 1/10 final dilution. The fortified tissue homogenates were analysed again and the type-specific and PAN-FMDV positivity were correctly revealed.

Moreover, an initial evaluation of the diagnostic sensitivity of the two multiplex LFDs was conducted by analysing a representative panel of vesicular epithelium suspensions (ES) from 21 outbreaks occurred in Tanzania, during 2012–2018, in which reference laboratory tests (VI and real-

time RT-PCR) had previously identified FMDV type O in five samples, type A in seven samples, SAT 1 in three and SAT 2 in six (Table 2). Samples confirmed positive for type O and A were analysed by the Eurasia LFD, while those recognized as containing SAT 1 and SAT 2 virus were analysed by the Africa LFD.

Results of the assessment and the comparison with the Ag-ELISA kit are summarized in Table 2. A complete concordance between results generated by the two assays was observed with the Eurasia LFD: one type O sample out of five and one type A out of seven were missed by both tests, in line with their lower sensitivity with respect to VI and PCR, while all other samples were correctly detected and typed. No cross-reactions between serotypes were observed, which attested the high specificity of the Eurasia LFD. Indeed, these 12 samples positive for FMDV-type O or A were contemporary tested also by the other two type-specific lines incorporated in the multiplex LFD generating a single coherent type-specific band, in addition to the PAN-FMDV reaction. Using the Africa LFD, the three SAT 1 samples were correctly typed by both Ag-ELISA kit and LFIA, providing similar reaction intensity in both the SAT 1-specific and in the PAN-FMDV test. Concerning the six SAT 2-positive samples, all were detected by the LFD type-specific lines, however one sample produced a very faint reaction and another one generated a cross-reaction with SAT 1. The SAT 2 type-specific ELISA test showed in general stronger reactions, while the SAT 1 cross-reactivity of the sample SAT2/TAN/2018–2 appeared far weaker. In contrast, the PAN-FMDV line in the Africa LFD recognized 5 out 6 samples containing a SAT 2 virus, resulting more performant than the PAN-FMDV test in the Ag-ELISA: the better performance of the LFD in this case is attributable to the contribution of both the type-specific and the PAN-FMDV mAbs used to generate the PAN-FMDV line, whilst the test is simply based on 1F10 mAb (PAN-FMDV, but poorly reacting with several SAT 2 strains) as both capture and detector antibody in the Antigen-ELISA.

Pictures of a few representative results observed when testing FMDV reference isolates or field samples with the Eurasia and Africa LFDs are shown in Fig. 3: test lines indicating the specific serotype of the virus are

4. Discussion

The study describes the first development of multiplexing LFDs aimed at the detection and typing of the five principal and more diffuse FMDV serotypes. Given the limitation in the number of reactive lines that can be accommodated in a single strip, two LFDs were designed taking into consideration the epidemiological distribution of FMDV se-rotypes in the target areas [3,4]. One LFD, for detecting and typing of O, A and Asia 1 serotypes, was named “Eurasia LFD” in line with the simultaneous circulation of these three serotypes in the wide region comprising the Asian continent, the Middle East and part of eastern Europe. This LFD, however, is also suited for African countries, where FMDV types O and A co-circulate with SAT types. The second device, named “Africa LFD”, enabled detection and differentiation of the SAT 1 and SAT 2 serotypes, generally confined to the African continent. Both devices also incorporated the PAN-FMDV line reporting on infection from FMDV, regardless the strain and the serotype involved, including eventually the serotype C, which was not specifically contemplated in LFDs because it is considered extinct [34], and serotype SAT 3 which is rarely occurring and confined to a restricted area in southern Africa. Accordingly, five reactive zones were arranged in the Eurasia LFD (three serotype-specific, one PAN-specific and a control line to ensure correct operation of the device) and four for the Africa LFD (two serotype-specific, one PAN-specific and the control line) to achieve a total of six simultaneous analyses.

FMDV is subject to continuous evolution, giving rise to extensive genetic and antigenic variation within each serotype. Therefore, the selection of suited immunoreagents, able to identify the variety of viral variants, is crucial for a correct diagnosis and typing. The mAbs used for the development of the two LFDs were fully characterized with respect to the essential features for diagnostic uses. In particular, for the type specific mAbs, selectivity towards a specific serotype associated with wide intra-serotypic detection was shown in previous studies [27,28] using a collection of FMD viruses representing different geographical origins and antigenic and molecular variation within each of the FMDV serotypes. Moreover, the combinations of capture and detector (GNP-conjugated) mAbs which were adopted to assemble the LFDs correspond to those previously selected and extensively validated for the updated version of the FMDV Antigen Detection and Serotyping ELISA Kit [27] that includes detection and typing of serotypes SAT 1 and SAT 2, in addition to O, A, C and Asia 1. Moreover, the PAN-FMDV mAb was previously exploited both in Antigen-ELISA kit and to set up a general LFIA, which was shown to be able to detect the seven FMDV serotypes with excellent concordance with the Antigen-ELISA [11].

In the design and distribution of the reactive lines, the PAN-FMDV reaction required particular consideration, due to the possible compe-tition of the mAb (#1F10) binding all FMDV serotypes with the type-specific mAbs for capturing the virus. In fact, differently from ELISA, where each mAb-antigen interaction occurs separately, in the multiplex LFIA platform all interactions occur almost simultaneouly; when two
clearly visible with absence of cross-reactions. The PAN-FMDV line in the Eurasia LFD (first line) strongly reacted with reference isolates of serotypes O, A, Asia 1 and C, as well as with field samples positive for type O or A (intensity consistent with virus load), while the intensity with SAT 1 or SAT 2 isolates/samples was dependent on both antigen concentration and the extent of SAT strains reactivity with the PAN-FMDV mAb 1F10. The PAN-FMDV line in the Africa device (third line) appears fainter in particular for serotypes O, A and Asia 1 due to the higher distance from the sample deposition compared to the Eurasia device. capture lines for the same virus are present (made by the cross-reacting and the type-specific mAb respectively), binding of the antigen to the first antibody line may subtract it from the binding to the others. For this reason, we designed the Eurasia device with the PAN-reactive line as the farthest from the sample application point. However, surprisingly this set up led to achieve poor sensitivity by the PAN-reactive test line. Starting from the experimental observation that the positioning of the PAN reactive line dramatically influenced the sensitivity, we investi-gated the possible reasons to justify the observation and suggested for the first time the existence of a new type of hook effect. When one mAb (targeting one epitope, repeated in several identical copies on the viral particle) is used as capture and detector ligand, the excess of detector mAb typically employed for increasing the sensitivity of sandwich im-munoassays, saturated the multiple epitope. This led to decreasing the amount of the viral antigen that could interact with the capture mAb through the same epitope and, ultimately, to limiting assay sensitivity.

The effect was exasperated by distancing the capture line from the sample application point, as the longer the path, the longer the time available for the detector-antigen complex formation. Then, we high-lighted the relevance of the ligand position onto the membrane in connection with the use of the same mAb as capture and detector ligand.

The gold standard test used for comparison of the analytical and diagnostic performances of the new devices was the Antigen-ELISA kit implemented with the same mAbs; both methods detect the same ana-lytes, i.e. viral antigens through evidence of specific antigen-antibody reactions, without the preliminary amplification of the target analytes that occurs in other tests, such as Virus Isolation and Polymerase Chain Reaction which are therefore intrinsically more sensitive.

The analytical and diagnostic performances resulted comparable to those of the reference Ag-ELISA kit, except for the serotype SAT 2 whose detection needs some improvement. The moderate sensitivity of the SAT 2 type-specific test line could be at least partly ascribed to the position of the line, which was necessarily farther from the sample application point compared to the SAT 1 line. Considering that both sandwich assays relied on the same mAb used as capture and detector reagent, the weaker signals at the second (farthest) line were expected.

The detection limit of the LFDs resulted in the range of 10^3-10^4 TCID50 /test depending on the serotype, when FMD viruses freshly grown in cell cultures were analysed. However, virus degradation is frequently observed in field biological samples affecting the perfor-mance of virus isolation; conversely, evidence was shown that the detection limit of LFDs in terms of TCID50 can improve of up to 100-1000-fold when testing clinical samples of vesicular epithelium which are not properly stored, thanks to the capability of immunoassays like LFDs or ELISAs to detect FMD viral antigens irrespective the virus integrity and infectivity.

Despite the inherent lower sensitivity of LFDs compared to genome amplification assays, a pilot evaluation on a few vesicular lesions confirmed that the novel LFDs provide a diagnostic sensitivity suffi-ciently adequate for a first line test for detection and confirmation of clinical cases and characterisation of serotypes. Indeed, it is in these biological samples (vesicular epithelium, saliva and oropharyngeal swabs sampled during the acute clinical phase) that the FMD virus usually reaches the concentration required to provide a positive signal in immunoassays.

LFDs represent the simplest tools for rapid on-site diagnosis of FMDV and the accessibility to such devices opens options for decentralised testing [35]. Indeed, thanks to their robustness and simplicity, these tests could be deployed and used on infected farms for in-field confir-mation of FMD in endemic countries affected by multiple serotypes or exposed to FMD virus incursions. In addition, these tests may become strategic components of contingency plans of FMD-free countries, as well as of a reagent bank to maintain preparedness and diagnostic capability. Validated LFDs have the potential to speed-up the confir-mation of suspect cases during an epidemic following disease intro-duction, contributing to promptly implement control measures.

Further studies for validation of the novel multiplex LFDs on clinical samples are in progress and will be described in a subsequent paper. The novel LFDs will be evaluated both on archival samples available at National and International Reference Laboratories, as well as in the field. At the same time, improvement of the FMDV SAT 2 serotype detection is under investigation. .

Credit author statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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