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New Route for Fast Detection of Antibodies against Zoonotic Pathogens in Sera of Slaughtered Pigs by Means of Flow-through Chemiluminescence Immunochips

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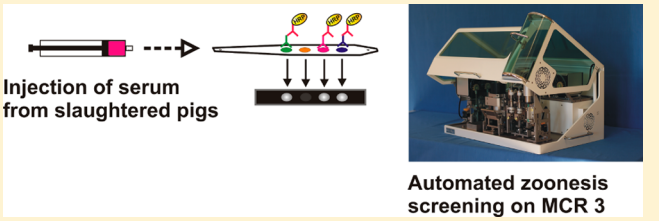
Supporting Information

ABSTRACT: The research on fast screening methods for antibodies against zoonotic pathogens in slaughter animals is important for food safety in farming and meat-processing industries. As a proof-of-concept study, antibodies against the emerging zoonotic pathogen hepatitis E virus (HEV) and enteropathogenic *Yersinia* spp. were analyzed in parallel using immobilized recombinant antigens (rAgs) of HEV genotypes 1 and 3 and Yersinia outer protein D (YopD) on a flow-through chemiluminescence immunochip. These rAgs are usually part of commercially available line immunoassays (LIAs) used for human diagnostics. In this study, sera from slaughtered pigs were tested on the microarray analysis platform MCR 3 to detect anti-HEV and anti-Yersinia IgG. The new method was characterized regarding signal reproducibility and specificity. The analytical performance was compared with in-house enzyme-linked immunosorbent assay (ELISA) and a LIA based on recomLine HEV (Mikrogen) or the ELISA test kit pigtype Yersinia Ab (Qiagen), respectively. The immunochip revealed the highest analytical sensitivity and was processed in 9 min automatically on the MCR 3. A comparative screening of swine serum samples from Bavarian slaughterhouses regarding anti-HEV and anti-Yersinia IgG seroprevalence was conducted. By using the LIA, 78% of the sera were tested positive for HEV antibodies. The immunochip and the ELISA identified anti-HEV IgG in 96% and 93% of the tested samples using the O2C-gt1 and O2C-gt3 rAg, respectively. The screening for anti-Yersinia IgG resulted in 86% positive findings using the immunochip and 57% and 48% for the ELISA methods, respectively, indicating a higher detection capability of the new method. Serum samples of slaughtered pigs could be analyzed faster and in an automated way on the microarray analysis platform MCR 3 which shows the great potential of the new immunochip assay format for multiplexed serum screening purposes.



Fast and automated detection methods for the analysis of seroprevalence of zoonoses in slaughter animals such as fattening pigs are highly demanded for monitoring and ensuring of good food hygiene and also for improving public health.[1,2] Zoonoses are infectious diseases that can be transmitted from animals, both wild and domestic, to humans. Zoonotic agents are, for example, bacteria, viruses, or parasites. Infected animals produce antibodies against these zoonotic pathogens. The blood sera of slaughtered pigs can easily be analyzed to determine the infectious status of the animal. In the case of porcine meat, the pathogens *Campylobacter* spp., *Yersinia* spp., *Salmonella* spp., *Trichinella* spp., hepatitis E virus (HEV), *Taenia* spp., and *Toxoplasma* spp. are of great interest for food safety and public health. Currently, only the screening for *Salmonella* spp. and *Trichinella* spp. is regulated by European law.[3,4]

HEV is an emerging pathogen and now considered as a zoonosis with domestic and wild pigs and more likely other species serving as animal reservoirs.[5,6] HEV is a nonenveloped, single-stranded positive-sense RNA virus classified in the family *Hepeviridae*.[7] HEV is composed of several genotypes (GTs), where GTs 1−4 are recognized as human pathogens. Human infections in developing countries are mostly associated with GTs 1 and 2, whereas GTs 3 and 4 are found more frequently in the industrialized world.[8] Studies concerning the distribution of HEV in mammalians discovered the virus in wild boar,[9,10] deer,[11] and also pigs.[6,12−14] These mammalians represent mainly a reservoir for GTs 3 and 4. Clinical symptoms in humans cannot be distinguished from other forms of viral

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hepatitis and are mostly self-limiting with generally low case fatality rates that may reach up to 25% in pregnant women.[15,16]

Enteropathogenic *Yersinia* spp. are enteroinvasive foodborne pathogens causing diarrheal disease in humans and are therefore of great interest in zoonosis monitoring.[17,18] Epidemiologic studies have demonstrated that pigs and pork serve as an important source for pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*.[19,20] The Yersinia virulence plasmid (pYV)-encoded type III secretion system (TTSS) is common to pathogenic Yersinia species, which require this TTSS to survive and replicate within lymphoid tissues of their animal or human hosts.[21] A set of pathogenicity factors, including those known as Yersinia outer proteins (Yops), is exported by this system during bacterial infection of host cells.[22] Yop antigens can be utilized for detection of pathogenic *Yersinia* spp. antibodies via affinity binding.

Common detection methods of viruses like HEV and diagnosis of acute infection in humans are immune electron microscopy (IEM),[23,24] polymerase chain reaction (PCR) assays,[25,26] and immunoassays.[27−29] By using immunoassay formats, host antibodies directed against the pathogen hosts are detected after binding to immobilized antigens. Since the infection with HEV and enteropathogenic Yersinia is asymptomatic in pigs, it is not routinely detected. In the case of HEV, first immunoassays based on enzyme-linked immunosorbent assay (ELISA) and line immunoassay (LIA) test strips are reported for the analysis of swine sera.[30,31] The common diagnosis of enteropathogenic *Yersina* spp. infection in pigs is culture and selective enrichment of feces or tonsil swabs, followed by PCR or pulsed-field gel electrophoreses (PFGE).[17,32,33] Also, immunoassays based on LPS proteins or Yops have been reported for detection in swine serum samples, and for example, the ELISA test kit pigtype Yersinia Ab (Qiagen) is commercially available.[34,35]

In our study, we tested the antibody reactivity in swine serum samples with recombinant HEV ORF2 antigens of GT 1 and GT 3 (O2C-gt1 and O2C-gt3) and recombinant ORF3 antigen of GT 3 (O3-gt3). These rAgs are immobilized either on a nitrocellulose test strip (LIA), polystyrene microtiter plates (ELISA), or immunochips for chemiluminescence microarray immunoassays (CL-MIA). For detection of anti-Yersinia IgG, we used the recombinant YopD antigen for in-house ELISA and immunochip measurements. The commercially available ELISA test kit pigtype Yersinia Ab (Qiagen) was used for comparative evaluation of the immunochip assay performance.

The microarray analysis platform MCR 3 (Munich chip reader 3rd generation) combines chemiluminescence readout with a flow-injection system.[36] Immunoassay methods require several incubation and washing steps. Flow-injection systems process the immunoassay automatically, which reduces manual operation steps. In comparison with static immunoassays (e.g., ELISA and LIA), flow-through immunoassays present thinner diffusion layers enabling efficient mass transport.[37] This significantly reduces the time needed to perform the assay. Thus, automated flow-through microarrays allow for the analysis of a sample within minutes and ensure reproducible and easy operating.

Analytical microarrays are a powerful tool for the simultaneous detection of multiple analytes.[38] Fluorescence-, electrochemistry-, and chemiluminescence (CL)-based detection methods are commonly used for readout purposes.[39,40] In CL microarray measurements, the light is emitted by an enzyme-assisted chemical reaction and can be recorded by a CCD camera. Due to low background signals, CL is the most sensitive readout principle for microarrays.[41,42]

CL analytical microarrays have been approved for the detection of DNA target molecules,[43] as well as for, for example, microorganisms,[44,45] food contaminants,[42,46,47] and toxins[48] or pharmaceuticals[49] in food and water samples. Antigen-based multiplexed arrays for detection of serum antibodies against different toxins and hepatitis B have been reported as well as microfluidic assays for analysis of allergen-specific or autoantibodies, showing the potential of this technique for antibody screening.[50−52]

For the principle study of swine serum analysis, the previously established surface chemistry of poly(ethylene glycol) (PEG) layers[53] was adapted and further optimized for the immobilization of rAgs. The generated immunochip was characterized by dilution experiments of serum samples and compared with LIA and ELISA measurements. The study was completed by testing serum samples from Bavarian slaughterhouses each with three immunoassay platforms regarding anti-HEV and anti-Yersinia IgG seroprevalences. The here-presented new route for fast and multiplexed screening of antibodies against zoonotic agents in sera of slaughtered pigs would reduce analysis time and costs and could be applied in future routine monitoring as a tailor-made solution for the meat producing industry.

MATERIALS AND METHODS

Sample preparation, ELISA measurements, and LIA measurements are standardized methods that were adapted for the comparison measurements. These methods and the compositions of the rAg buffer solutions used for immobilization are described in detail in the Supporting Information.

Immunochip Production. Commercially available polystyrene microscope slides were tested without any additional modification. Before use, the slides were treated in methanol for 5 min by sonication. Two activation methods of diamino-PEGylated (DAPEG) glass slides were studied regarding performance and efficacy for covalent immobilization of rAgs. The fabrication of DAPEG-coated slides is based on the in-house protocol published formerly.[53] Details of the preparation are given in the Supporting Information.

These amino-PEG slides were used for further functionalization. For covalent immobilization of the HEV antigens, terminal amino groups should be addressed for efficient binding on the chip surface. Thus, two common surface activation strategies were compared: the introduction of a reactive epoxy group, resulting in an epoxy-PEG-surface, and the linkage of protein and surface via *N*-hydroxysuccinimide (NHS) activation (preparation protocols are presented in the Supporting Information).

For immobilization, HEV and Yersinia antigen stock solutions were thawed and directly used for spotting. Also, antigen samples were diluted with MOPS storage buffer and then used for immobilization. For experiments regarding buffer conditions, 50 μL samples were transferred to Amicon filter units (0.5 mL) and diluted with 450 μL of coating buffer as used for microtiter plate experiments, additionally containing 0.01% (v/v) Tween-20. Buffer exchange was performed following the manufacturer's advice by centrifuging for 15 min (14 000 rpm, 4 °C) and recovering for 2 min (1000 rpm, 4 °C) (Zentrifuge Universal 320R; Hettich, Tuttlingen, Germany).

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Immobilization of rAgs was performed by contact printing with a BioOdyssey calligrapher miniarrayer from Bio-Rad Laboratories (Munich, Germany) using the Stealth solid pin SNS 9 from ArrayIt (Sunnyvale, CA, USA). For spotting, approximately 35 μL of each antigen solution was transferred to the cavities of a 384-well PP flat bottom microtiter plate and spotted on the prepared activated glass or polystyrene slides, respectively. Two clusters were set on one microarray chip with a grid spacing of 1300 μm for the columns and 1100 μm for the rows, respectively. Each solution was spotted in five replicates. During the spotting process, the slides were cooled to 20 °C, and the humidity in the spotting chamber was set to 50%. After spotting, the microarray chips were incubated for 15 h at 25 °C and 50% humidity. The deactivation of free reactive binding sides was carried out by gently shaking the slides in an aqueous buffer containing 1 M Tris, 150 mM sodium chloride, and 0.05% (v/v) Tween-20 buffer (adjusted with hydrochloric acid to pH 7.8) for 15 min. To minimize adsorption of serum matrix proteins, a succeeding blocking step with 1% (w/v) bovine serum albumin (BSA) in PBST (10 mM potassium dihydrogen phosphate, 70 mM dipotassium hydrogen phosphate, 145 mM sodium chloride, 0.05% (v/v) Tween-20) followed for 30 min. Finally, the slides were rinsed three times with PBST and cleaned by shaking in PBST for 15 min.

After drying under a continuous nitrogen flow, the glass slides were connected with plastic carriers presenting in- and outlets by use of a double-sided adhesive foil forming the two microfluidic measuring channels of one immunochip.

Immunochip Measurements. The computer-controlled protocol of the CL microarray immunoassay (CL-MIA) could be summarized as following: 1000 μL of the diluted serum sample was injected automatically in the microfluidic system using a plastic disposable sample syringe. Thereby, 100 μL of the sample was pumped directly to the waste reservoir in order to prevent air bubbles intruding into the flow channel. Because of the capillary dead volume, 200 μL of the sample was pumped to the measurement channel at a high flow rate of 100 μL/s, followed by 700 μL at a flow rate of 10 μL/s. Afterward, 1000 μL of running buffer was given over the chip at a flow rate of 10 μL/s to ensure that the complete sample amount was pumped over the chip surface. After a washing step (2000 μL of running buffer, 500 μL/s), the detection antibody solution was added. Therefore, the first 200 μL of antiswine IgG–HRP conjugate (1 μg/mL) at a flow rate of 100 μL/s and then 800 μL at 10 μL/s was disposed. During addition of the detection antibody, the sample injection unit was rinsed intensively with running buffer to avoid cross contamination. After a second washing step of the chip surface (2000 μL running buffer, 500 μL/s), a mixture of each 200 μL of CL substrate was given over the chip at a flow rate of 150 μL/s. The flow was stopped, and a picture was taken for 60 s by the CCD camera. After recording, the whole microfluidic system was rinsed thoroughly and cleaned with running buffer to prepare it for the next measurement. All immunochemical assay steps including cleaning of the capillaries took 8.5 min. Due to the implementation of two flow channels on one microarray chip, two measurements could be performed with one chip. The measurements on the MCR 3 platform result in two-dimensional (2D) images (2 × 2 pixel binning mode, 696 × 520 pixels) of the chip surface obtained by a 16-bit CCD camera. The resolution of one pixel is approximately 40 μm. The images of the CCD camera were automatically saved as text files. Before each measurement, a background picture was taken. This background blank was automatically subtracted from the measuring images. CL signals were evaluated with the image evaluation software MCRImageAnalyzer (GWK GmbH, Munich, Germany) developed for the automated data-processing of CL microarrays. Details of data evaluation are given in a former publication.[47] The calculated CL data were transferred to Origin 7.0 (MicroCal Software Inc., Newark, NJ, USA) for graphical evaluation.

RESULTS AND DISCUSSION

Characterization of Immunochip Performance. Two different activation methods of diamino-PEG (DAPEG)-coated glass slides were investigated (schematic illustration of surface modification is presented in the Supporting Information). On the one hand, activation with introduction of reactive terminal epoxy groups was tested (epoxy-PEG surface), and on the other hand, an activation method via an NHS ester was applied (NHS-amid-PEG surface). Both strategies introduce functional groups to react with primary amine residues of the rAgs. To compare this covalent attachment with immobilization based on adsorption, Nunc polystyrene microscope slides were tested. The optimization results of the rAg immunochips are described and discussed in detail on the example of HEV rAg O2C-gt1 in the Supporting Information. The immobilization efficacies of the three surface modifications were evaluated with the MOPS-SDS stock solution of O2C-gt1. Therefore, a positive serum sample was used (H 12). Comparing covalent and adsorptive binding of antigen and antibody to the surface, the functionalized glass slides revealed significantly higher signals than the polystyrene surface, confirming that the covalent coupling of terminal amino groups to the activated chip surface is the preferred immobilization strategy also for HEV recombinant antigens (see Table S-1, Supporting Information). Epoxy-PEG-activated chips showed the highest CL signals for the positive control and O2C-gt1 antigen, presenting similar background signals for the negative control spots. Furthermore, the lowest signal variations could be achieved for the epoxy-PEG surface. Hence, epoxy-PEG microarray chips were selected for further studies.

In order to prove repeatability and reproducibility, several microarray chips were analyzed on the same measurement day and also on different measurement days. This procedure included different batches of diamino-PEGylated glass slides used for activation and spotting process. As described in the Supporting Information, also the preparation of the spotting solution for HEV O2C-gt3 had to be done at each measurement day. The CL signals were evaluated for three measurement days regarding spot quality (intra-assay variation) and chip-to-chip variation (interassay variation). The results of measurements for both HEV antigens are depicted in Figure S-4 (Supporting Information), and coefficients of variation (CVs) are listed in Table S-2 (Supporting Information). Low intra-assay CVs in the range of 5% could be revealed for all microarray measurements, which indicates a high homogeneity of the epoxy-PEG surface and suitability of spotting buffer composition. For O2C-gt1, evaluation of the CL signal and interassay variation in reference to spotting concentration lead to the conclusion that 1:2 dilution of the antigen stock solution improves the signal reproducibility, maintaining comparable signal values. The statistical parameters for HEV antigen O2C-gt1 show high reproducibility of the chip preparation and CL-MIA performance. Taking in consideration that all reagents are pumped in continuous flow where no equilibrium state is attained, the low overall interassay CV of 6.6% (n = 33)

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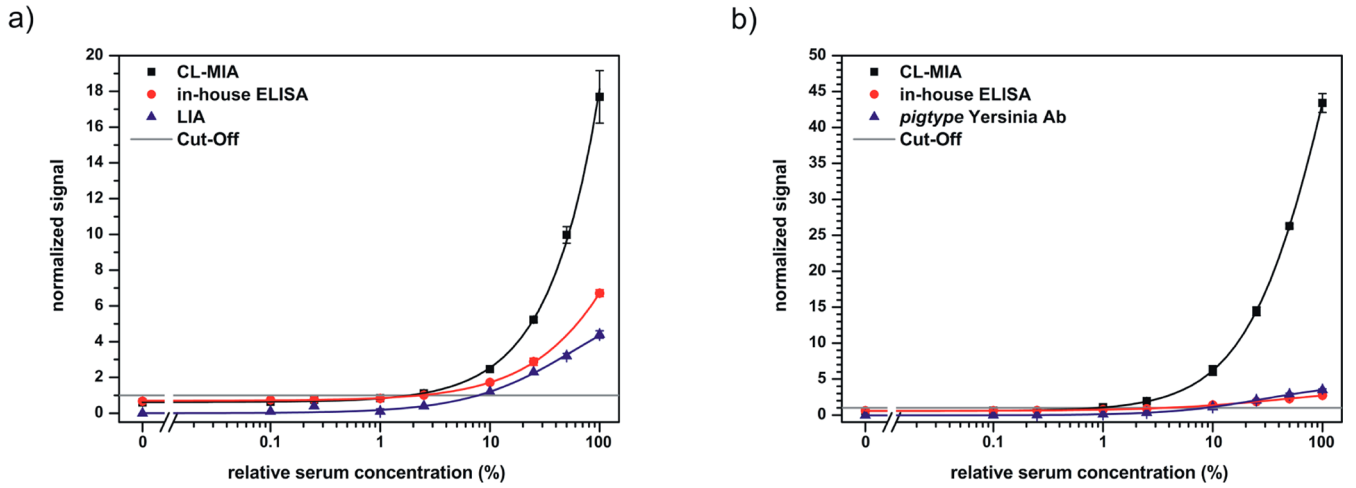

Figure 1. Illustration of dose−response measurements for (a) HEV antigen O2C-gt1 and (b) Yop antigens using CL-MIA (□, black), in-house ELISA (○, red), and LIA strip or pigtype Yersinia Ab, respectively (Δ, blue). Data are plotted using logistic fit (with a) CL-MIA, LIA: n = 9; in-house ELISA: n = 8, and (b) CL-MIA: n = 8; in-house ELISA, pigtype Yersinia Ab: n = 9. Error bars represent interassay variation (1 s; CL-MIA, LIA: m = 2; ELISA: m = 3), straight line (gray) represents cutoff.

confirms the feasibility of this newly developed rAg-based detection method. Because of this result, the introduction of an interval of prediction, calculated for P = 0.95, is an appropriate tool to characterize the assay quality and to assume future measurements for serum screening purposes. The immunochip based on immobilized rAg O2C-gt1 presents a suitable and fast detection method for HEV IgG antibodies in serum samples. In contrast, the signals plotted for O2C-gt3 show higher variations. This trend can be explained by the stability concerns of the recombinant antigen as mentioned in the Supporting Information. The reduction of the SDS content by a factor of 10 ensures antigen reactivity but implicates higher chip-to-chip variations. However, the repeatability on one measurement day could be calculated in this study to 7−11%, which is an acceptable performance for immunoassays with a complex matrix. In terms of reproducibility over several measurement days, the CL signals for O2C-gt3 are affected by the necessity of daily buffer exchange.

■ DILUTION EXPERIMENTS

In human diagnostics, the analytical method for marker detection is correlated to clinical diagnosis given by definite symptoms. This allows for definition of positive and negative samples according to specificity and sensitivity tests with healthy and nonhealthy patients in clinical studies. In contrast, most infections in pigs caused by zoonotic agents, which may lead to foodborne infection in humans, show an asymptomatic course of disease. Thus, supporting immunoassay methods were needed to compare the screening results of the newly developed immunochip. This was realized by means of the line immunoassay recomLine HEV IgG/IgM (Mikrogen), which is commercially available for human diagnostic, and the ELISA test kit pigtype Yersinia Ab (Qiagen). By using the antiswine IgG−HRP conjugate, the LIA was adapted for analysis of swine serum samples. For discrimination of positive and negative samples, the recomLine HEV test strips imply a cutoff band on which the specific antigen bands are normalized after color development. Samples with antigen signal intensities equal or higher than the cutoff signal (cutoff intensity, COI ≥ 1.0) were assessed as positive. The pigtype Yersinia Ab test includes positive and negative controls, and by following the manufacturer's instructions, the cutoff level was set to be at a sample-to-positive control ratio (S/P ratio) of 0.3.

To establish a cutoff level for the immunochip method, repeated measurements of blank samples were conducted to prove the assay performance. A real serum sample showing no signal in the LIA (COI = 0.0) was selected as the blank sample (H 27) for determination of the cutoff level for anti-HEV IgG. For anti-Yersinia IgG, the sample Y 21 was used for blank measurements due to the negative result using the pigtype Yersinia Ab test. In analogy to the test of reproducibility, measurements of the blank sample were performed on three different days to reflect the variability of chip preparation and immunoassay. In Figures S-5 and S-6 (Supporting Information), the results of this interday study are illustrated for O2C-gt1, O2C-gt3, and YopD, respectively. In accordance with the results of the assay characterization, a high reproducibility of the blank measurements for O2C-gt1 could be demonstrated by intraday CV of 3.9% (n = 15), 5.5% (n = 6), and 4.9% (n = 5) for single days. An overall interday CV of 4.5% (n = 26), corresponding to an absolute blank CL signal of 586 ± 26 au, could be determined. For O2C-gt3, the CV values of the single days also show high repeatability (4.3%, 4.5%, and 5.7%). Discussing the absolute values, slightly bigger deviations of the single days mean values were obtained, which can be expressed by an overall CV of 7.8% or an absolute blank CL signal of 612 ± 48 au, respectively. Evaluation of the inter-assay variation of blank measurements based on YopD lead to intraday CVs of 13.3% (n = 6), 8.5% (n = 6), and 6.1 (n = 8) resulting in an overall interday CV of 9.7% (n = 20). This corresponds to an absolute blank CL signal of 499 ± 48 au, which provides a sufficient assay quality for serum screening purposes regarding anti-Yersinia IgG.

For assessment of serum samples, an analytical cutoff was calculated based on these blank measurements. Therefore, the cutoff was set to be at the signal level of the mean blank signal added by 10-fold standard deviation to ensure avoidance of false-positive findings regarding specific antibody presence in the sample. With this cutoff definition, dose−response measurements could be compared after normalization by determination of that antibody concentration that falls below the cutoff limit.

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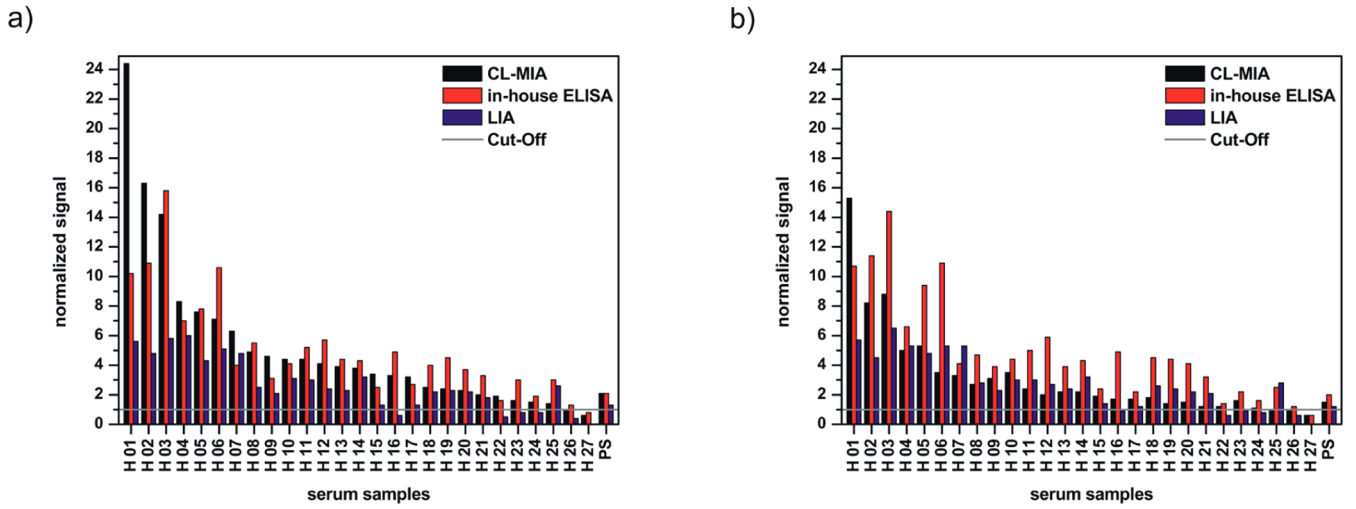

Figure 2. Results of serum screening regarding anti-HEV IgG using different assay platforms based on HEV antigen (a) O2C-gt1 and (b) O2C-gt3. CL-MIA (black), in-house ELISA (red), and LIA strip (blue) were performed, and signals were normalized on cutoff level (straight line, gray). Serum samples were labeled and arranged with respect to their CL signal for O2C-gt1.

For anti-HEV IgG, a high-positive serum sample (H 02) was diluted with blank serum (H 27) to 1:2, 1:4, 1:10, 1:40, 1:100, 1:400, and 1:1000. In analogy, for screening of Yersinia IgG, the highly anti-Yersinia IgG-positive sample Y 01 was diluted with blank serum Y 21.

f1 In Figure 1a, representative results of dilution experiments for HEV antigen O2C-gt1 are depicted for CL-MIA, LIA, and ELISA. The results of the dilution experiments based on Yop antigens are shown in Figure 1b. Immunochip measurements were performed with both measuring channels of each microarray chip, and LIA measurements were also executed in duplicate with two test strips per serum dilution. The plots visualize the high sensitivity and large dynamic range of the developed immunochip on the analysis platform MCR 3 compared to the LIA and ELISA tests, allowing for more precise discrimination of serum samples in regard to their IgG antibody level. Considering the cutoff levels, a 4-fold higher dilution could be evaluated with in-house ELISA and immunochips in the case of anti-HEV IgG. Comparing the dose–response curves for detection of anti-Yersinia IgG, the immunochip method even allows for identification of antibodies at a 10-fold higher dilution factor than the microtiter plate-based assays.

Simultaneously, the immunochip showed the best response of the normalized signal to increasing antibody levels. Hence, an effective differentiation of anti-HEV or anti-Yersinia IgG positive and negative swine serum samples on the analysis platform MCR 3 is possible within less than 9 min.

Screening Experiments with Swine Sera. To conclude the characterization of the rAg immunochip, the applicability of the new method for detection of HEV and Yersinia IgG antibodies was investigated by screening and assessment of real serum samples. Therefore, 27 serum samples (H 01 to H 27), collected at Bavarian slaughterhouses, were tested with immunochips, in-house ELISA, and porcine recomLine HEV based on recombinant antigens O2C-gt1 and O2C-gt3. Twenty one samples (Y 01 to Y 21) were tested with immunochips and in-house ELISA based on recombinant YopD and additionally the commercially available pigtype Yersinia Ab ELISA test, which also uses immobilized Yop antigens. Furthermore, a commercially available pooled serum sample (PS), originated from a French slaughterhouse, was analyzed. The immunochip measurements were executed on different days. The results of the serum screening are illustrated in Figures 2 and 3. With f2f3

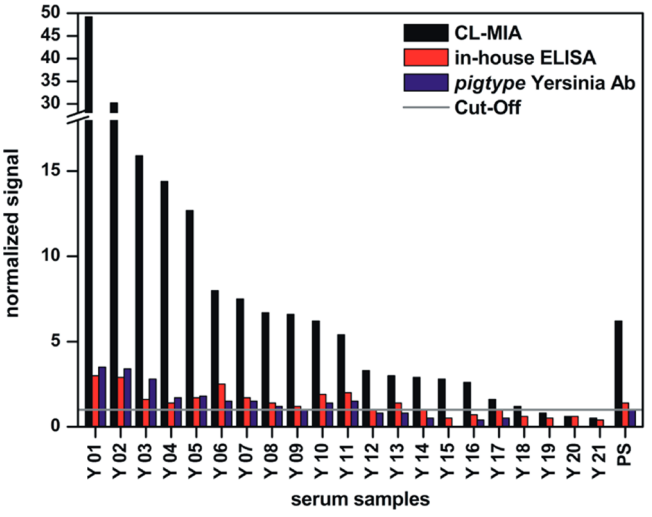

Figure 3. Results of serum screening regarding anti-Yersinia IgG using different assay platforms based on Yop antigens. CL-MIA (black), in-house ELISA (red), and pigtype Yersinia Ab (blue) were performed, and signals were normalized on cutoff level (straight line, gray). Serum samples were labeled and arranged with respect to their CL signal.

reference to the cutoff level (COI = 1.0), samples could be assumed to be "positive" or "negative". By comparing the assay signals, the large dynamic range of the immunochip could be confirmed by the significant signal differences between samples with higher (S 01 to S 06, Y 01 to Y05) and lower antibody content, respectively.

In general, similar signal trends could be found for both HEV antigens, although lower reactivity of the O2C-gt3 antigen is visible. 78% swine sera were tested positively using the LIA. The immunochip and the in-house ELISA identified HEV antibodies in 93% and 96%, respectively, of the tested swine sera using the O2C-gt1 and O2C-gt3 rAg. Taking the results of the dilution measurements in consideration, the lower limit of

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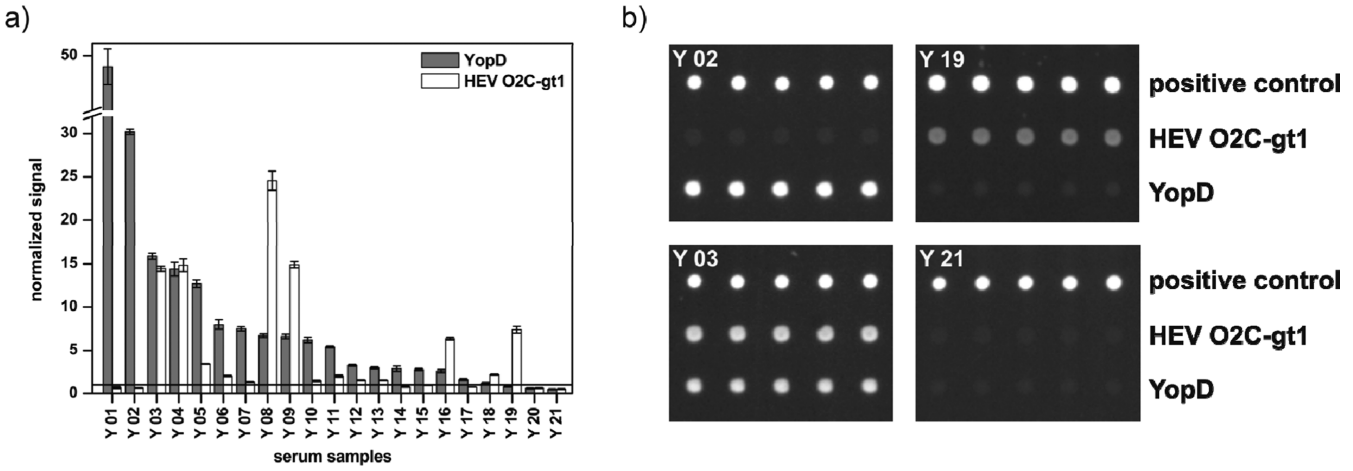

Figure 4. Results of multiplexed swine serum screening regarding anti-Yersinia and anti-HEV IgG: (a) column plot of immunochip measurements based on YopD (gray) and HEV O2C-gt1 (white). Signals were normalized on cutoff level (straight line, black). Error bars represent intra-assay variation of five replicate spots (1 s, $m = 5$); (b) representative 2D images of immunochips, incubated with different swine serum samples, after CL readout.

detection proven for the immunochip presents a plausible explanation for this finding. Furthermore, all samples tested positive for HEV IgG in the LIA could be confirmed to be positive with immunochip and ELISA measurements.

The evaluation of the sample screening regarding anti-Yersinia IgG confirms the potential of the immunochip for sensitive antibody screening purposes: serum samples with lower antibody content (Y 12 to Y 18), showing signals in the range or below the cutoff level in both microtiter plate assays, could be still assessed as positive using the MCR 3. For the immunochip assay, 86% of the swine serum samples were tested positively, whereas by using in-house ELISA and pigtype Yersinia Ab, 57% and 48%, respectively, of the samples resulted in positive findings. This obvious difference in the obtained seroprevalence can be explained by the higher detection capability of the immunochip method.

For detailed investigation regarding the feasibility of the introduced cutoff definition, more samples with low antibody content have to be analyzed in future studies. The analysis of the commercially available pooled serum sample PS resulted in positive findings for all assay platforms.

To complete the characterization of the new immunochip, the applicability of the method for multiplexed screening of swine serum samples was tested by preparing microarray chips on which the recombinant antigens HEV O2C-gt1 and YopD were both immobilized. With these immunochips, the serum samples Y 01 to Y21 were screened simultaneously for anti-HEV and anti-Yersinia IgG. The results of the measurements are illustrated in Figure 4a, and representative images obtained by the CCD camera after CL readout are depicted in Figure 4b. It could be shown that differentiation between the target antibodies is possible. Since evaluation of samples Y 02 and Y 19 resulted in signal only for one of the recombinant antigens, high specificity toward their targets and low cross reactivity can be reasoned. At the same time, measurement of sample Y 21 showed that no unspecific binding of serum matrix components on the spot surfaces took place. Thus, this successful first proof of the multiplex approach constitutes the great potential of the newly developed rAg-based immunochip for application as a rapid, automated, and specific screening method of a broad variety of antibodies against zoonotic diseases in swine serum samples.

CONCLUSIONS

In this study, we have shown that swine sera could be screened rapidly and automatically for anti-HEV and anti-Yersinia IgG using rAg-based immunochips on the analysis platform MCR 3 in parallel. For this purpose, HEV rAg O2C-gt1 and O2C-gt3 and YopD were immobilized on epoxy-PEG-functionalized glass slides. Since the porcine HEV LIA and the in-house ELISA showed complete agreement of the serum screening based on both HEV antigens and due to known high cross reactivity of O2C-gt1,[31] the immunochip design using O2C-gt1 is suitable for detection of HEV IgG antibodies in swine serum matrix. The immunochip measurements for determination of anti-Yersinia IgG using YopD confirmed the potential of the new method featuring a higher detection capability than the tested in-house ELISA and the commercially available ELISA kit pigtype Yersinia Ab (Qiagen).

The next steps in order to establish a fast screening method for sera of slaughtering pigs are to integrate immobilized antigens of *Salmonella* spp. and *Toxoplasma* spp. on the immunochip and to investigate possible antigen cross reactivity in more detail. A multiplexed screening of serum samples regarding relevant zoonotic infectious diseases could be performed within a few minutes. Such a fast, automated, multiplexed, and tailor-made detection method would aid the meat-producing industries concerning food safety and public health aspects.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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