Detection of Individual Microbial Pathogens by Proximity Ligation

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**Background:** Nucleic acid amplification allows the detection of single infectious agents. Protein-based assays, although they provide information on ongoing infections, have substantially less detection sensitivity.

**Methods:** We used proximity ligation reactions to detect proteins on bacteria and virus particles via nucleic acid amplification. Antibodies recognizing viral or bacterial surface proteins were equipped with DNA strands that could be joined by ligation when several antibodies were bound in proximity to surface proteins of individual infectious agents.

**Results:** Detection sensitivities similar to those of nucleic acid-based detection reactions were achieved directly in infected samples for a parvovirus and an intracellular bacterium.

**Conclusions:** This method enables detection of ligated DNA strands with good sensitivity by real-time PCR and could be of value for early diagnosis of infectious disease and in biodefense.

Infections account for more than 10 million deaths worldwide annually and contribute to 15% of human cancers (1–4). PCR has revolutionized infectious disease diagnostics and research, but often, information is needed about the concentrations of pathogen proteins. Because more protein antigens than nucleic acids are produced during infection, methods based on protein analysis can be more sensitive than nucleic acid–based detection. Assays based on pathogen genome amplification cannot determine viability; PCR analysis of infectious prions, which lack nucleic acid components, is impossible; and strain variability, limited multiplexing capacity, and complex sample materials are additional problems not easily solved by nucleic acid–based detection. Methods that detect viral proteins that exist only in the acute phase of the disease provide valuable information (5), and HIV structural proteins and glycoproteins can be detected in the germinal center of lymph nodes in the absence of detectable virus replication in patients undergoing highly active antiretroviral therapy (6). Sensitive protein-based microbial detection can provide valuable information about ongoing infection, and it is an important complement to nucleic acid–based detection.

The proximity ligation assay (PLA)⁶ (7, 8) enables sensitive detection of proteins, for example, cytokines, in complex biological samples (8–10). In PLA, affinity probes that bind target proteins are equipped with DNA strands that can be joined by ligation when 2 or more such reagents are brought into proximity by binding to the same target molecule. The DNA ligation products are subsequently detected by DNA amplification. The reaction is performed in 3 steps (Fig. 1): (a) samples are incubated with pairs of proximity probes that consist of target-specific antibodies with conjugated oligonucleotides; (b) reagents necessary for ligation and amplification by PCR are added, allowing the free 5’ and 3’ ends of oligonucleotides on pairs of probes bound to the same target to be hybridized to a common connector oligonucleotide and joined by a DNA ligase; and (c) the ligation products are amplified with real-time detection of the accumulating DNA strands. The reactions can be performed as convenient homogeneous assays requiring only addition of reagents without washes. In an alternative solid-phase variation, the target is first bound to immobilized antibodies, allowing potentially interfering sub-

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¹ Nonstandard abbreviations: PLA, proximity ligation assay; PPV, porcine parvovirus; mAb, monoclonal antibody; TCID, tissue culture infectious units or particles; PBS, phosphate-buffered saline; HA, hemagglutination; qPCR, quantitative PCR; HRP, horseradish peroxidase; and Cₚ, threshold cycle.
stances and excess reagents to be removed by washes (Fig. 2) (7). Detection of *Bacillus anthracis*, *Bacillus subtilis*, and *Bacillus cereus* spores by homogeneous PLA with “burrs”, conjugates of peptide aptamers raised against the spore surface, oligonucleotides, and phycoerythrin, was recently reported (11).

We used PLA to detect 2 different infectious organisms in biological samples, the porcine parvovirus (PPV) and the bacterium *Lawsonia intracellularis*. PPV, a major cause of reproductive failure in swine, is transmitted horizon-

Fig. 1. Homogeneous-phase PLA performed in 3 steps.

(A), a 1-μL microbe sample is incubated with a pair of proximity probes in a volume of 5 μL for 1 h at 37 °C. The proximity probes are antibodies conjugated with oligonucleotides having either a 5′ (gray ribbon) or 3′ (black ribbon) end. (B), a common ligation and amplification mixture (45 μL) is added. The free 5′ and 3′ ends of proximity probes bound to the same microbe target hybridize to a common connector oligonucleotide and are ligated by T4 DNA ligase. (C), the ligation products of the proximity probes are amplified and detected by qPCR.

Fig. 2. Solid-phase PLA.

(A), a microbial target is bound to an immobilized antibody in a microtiter well, and unbound particles and other sample components are then removed by washes. (B), proximity probes are then added to the well. After incubation for 1 h at 37 °C, proximity probes that have not bound to the target are washed away. The common ligation and amplification mixture is added, and proximity probes that have bound the target in pairs are ligated and amplified with real-time detection and quantification of the products.
tally by contact with both respiratory and excretory secretions. Clinical signs of infection are usually absent, but the virus can be detected in organs or body fluids from an affected fetus. L. intracellularis, the causative agent of proliferative enteropathy in swine, is a gram-negative, obligate intracellular bacterium belonging to the Desulfovibrionaceae family. L. intracellularis infections in pigs are associated with proliferative lesions of the mucosa of the ileum and large intestine, and the bacterium can be isolated from fecal samples or from intestinal contents (12–14).

Materials and Methods

PROPAGATION AND PURIFICATION OF PPV
PPV strains NADL-2 and the anti-PPV monoclonal antibody (mAb) 5B were used in this study. The virus had a titer of $10^8$ tissue culture infectious units or particles (TCID$_{50}$/mL) and was propagated in PK-15 cells in roller bottles as reported by Rivera et al. (15). mAb 5B was produced at Svanova Biotech AB.

Supernatants of virus-infected cell cultures were clarified by low-speed centrifugation. Virus particles were then concentrated by pelleting with an ultracentrifuge (Sorvall$^\text{TM}$; Thermo Electron Corp.) at 16 000g for 4 h. The pelleted virus was resuspended in phosphate-buffered saline (PBS containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na$_2$HPO$_4$, 2 mmol/L KH$_2$PO$_4$), layered on a linear 20%–60% sucrose gradient, and centrifuged at 100 000g for 1 h. The white band containing the virus was collected and kept at $-70^\circ$C until used. This partially purified virus was used as an immunogen for the preparation of mAb 5B.

PROPAGATION AND PURIFICATION OF L. intracellularis
The L. intracellularis strain EU-01 was kindly provided by Dr. Keller, Bioscreen, Munich, Germany. The bacterial cultivation was prepared by inoculation of the bacteria on McCoy cells. The bacterial suspension used as a standard throughout the experiments had a titer of $10^6$ TCID$_{50}$/mL. The cultivation and titration were performed as described previously by Kroll et al. (16). We used 5 anti-Lawsonia mAbs, 110:9, 287:6, 268:18, 268:29, and 113:2. The antibodies were produced at Svanova Biotech AB.

The bacteria in the supernatant were concentrated by centrifugation at 37 000g for 15 min. The pellet was resuspended in Percoll (Pharmacia), and the solution was centrifuged at 37 000g for 1 h. The white band containing L. intracellularis was collected. This material was used as an immunogen for preparation of the mAbs.

HEMAGGLUTINATION TEST
The hemagglutination (HA) test was performed as described by Rivera et al. (17).

FIELD SAMPLES
PPV. A total of 10 PPV-positive and 10 PPV-negative samples were used throughout this study. The samples were randomly selected and originated from a larger study in which immunized and nonimmunized pregnant gilts were exposed to PPV. Tissue samples were collected from fetuses and were classified as PPV positive or negative after we assayed them with different techniques, such as the HA test, immunofluorescence assay, and nested PCR. Details regarding immunization procedures and results from the challenge tests have been reported previously by Bélation et al. (18) and Rivera et al. (15).

L. intracellularis. A total of 10 Lawsonia-positive and 10 Lawsonia-negative fecal samples collected at pig farms in Sweden were used. Most of the positive field samples were kindly supplied by Dr. Magdalena Jacobsson at the University of Agricultural Sciences in Uppsala, Sweden. The fecal samples were classified as positive or negative for Lawsonia by an in-house capture ELISA (Svanova Biotech). The samples were prepared by making a 1:10 suspension in PBS (100 µL of sample in 900 µL of PBS). The supernatants were then used for the PLA assay as well as the quantitative PCR (qPCR) and capture ELISA.

PREPARATION OF mAbs
Six BALB/C mice were immunized subcutaneously twice, 6 weeks apart, with 70 µg of purified PPV virus or L. intracellularis bacteria that was mixed with an equal volume of Freund’s complete or incomplete adjuvant. The animals were sacrificed 4 days after the second immunization. Spleen cells were collected and fused with the myeloma cell line SP 2/0 (19). Supernatants from the hybridoma cell clones were tested by an indirect ELISA with purified virus or bacteria as coating material. Bound antibodies were detected by a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Dakopatts) diluted 1:1000. Positive clones were subcloned several times, restested by ELISA, and stored at $-135^\circ$C. Selected clones were cultured, and the produced mAbs were purified as described previously (20).

BIOTINYLANALYSIS OF mAbs
We performed biotinylation according to the manufacturer’s instructions (Roche Diagnostics Corp). Briefly, N-hydroxysuccinimide ester was mixed with the antibody in a 40-fold molar excess and with a volume ratio of 1:10. The mixtures were incubated for 4 h at room temperature under constant mixing. The biotinylated mAbs were then dialyzed extensively against PBS to remove any unbound biotin.

FUNCTIONALITY TEST OF THE BIOTINYLATED mAbs
The biotinylated mAbs were tested with a commercially available PPV ELISA (Svanovir® PPV-A test) (21) and an in-house Lawsonia capture ELISA (Svanova Biotech) according to the manual for the assay or the in-house method, respectively, but with the following exceptions. The biotinylated PPV/Lawsonia mAbs were used instead of the HRP-conjugated mAb included in the standard test system. To visualize the binding of the biotinylated mAbs, HRP-labeled streptavidin (Dakopatts) was used in a dilu-
tion of 1:5000. The absorbance in the wells was measured with a spectrophotometer (Flow Laboratories) set at 450 nm.

PREPARATION OF PROXIMITY PROBES

Proximity probes were prepared according to Gullberg et al. (8). Thiol-modified oligonucleotides were coupled to maleimide-derivatized streptavidin, creating streptavidin–oligonucleotide conjugates with free 3' and 5' ends, respectively (5'-freeSTV, 5'-P-TCGTGTCAAAAAGCTCGTTACCTTGATTCCTAAACTCCTCT CGTAAGGAATTCCGGG CATCGGTGA-3'; 3'-freeSTV, 5'-CGCATCGCCCTTGGACTACGTAGACGACAGGCTTGGCCTGACTGACGGC CTAAATCGTG-3'OH). The biotinylated mAbs were combined with the streptavidin-oligonucleotide conjugates as follows: The biotinylated antibodies were diluted in PBS containing 10 g/L bovine serum albumin (Sigma) to a final concentration of 30 nmol/L. The antibodies were then combined with the streptavidin–oligonucleotide conjugates in a 1:1 ratio in a volume of 5 µL at room temperature for 1 h. The antibody–oligonucleotide probes were then diluted to a concentration of 1.2 nmol/L in a probe dilution buffer [PBS containing 10 g/L bovine serum albumin, 16 mg/L sheared polyA bulk nucleic acid (Sigma), and 1 mmol/L d-biotin (Molecular Probes)] and stored at 4 °C until use.

DETECTION OF PPV AND L. intracellularis BY SOLUTION-PHASE PLA

In the homogeneous PLA, 1-µL samples were incubated in optical PCR tubes (Applied Biosystems) at 37 °C with 4 µL of a mixture containing the proximity probe pairs, each diluted to a concentration of 24 pmol/L in the probe dilution buffer. After 1 h of incubation, 45 µL of a combined mixture for ligation and amplification was added [final concentrations, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.15 mM MgCl2, 0.4 Weiss units of T4 DNA ligase (Fermentas), 400 nM connector oligonucleotide (5'-TACCTTGATTCCCCTAACCCTGATGAAAAATTCGG-3'; biomers.net GmbH), 80 µM ATP, 200 µM each of the deoxynucleoside triphosphates, 100 nM primers (forward, 5'-CA-TCCGCCCCTTGACTACGA-3'; reverse, 5'-GGGAATCAGGTAACCGGACTTAGG-3'; biomers.net GmbH), 100 nM TaqMan® MGB probe (5'-FAM-TGACGAACCGCCTT-GCATGACTGAMG-3'; where FAM is 6-carboxyfluorescent and MGBNFQ is major groove binder with a nonfluorescent quencher; Applied Biosystems), and 1.5 U of Platinum Taq DNA polymerase (Invitrogen)]. After addition of the combined mixture, the tubes were sealed with optical PCR lids (Applied Biosystems) and transferred to a real-time PCR instrument (MX 3000P from Stratagene, or ABI 7700 or ABI 7000 from Applied Biosystems). The reactions were incubated at 95 °C for 2 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The results are presented as threshold cycle (Ct) values; as signal-to-noise ratios, for which the number of ligations of proximity probes pairs that occurred in the sample were divided by the number of ligations in the negative control; or as infectious doses, for which the data were correlated to a standard dilution series.

DETECTION OF PPV AND L. intracellularis BY SOLID-PHASE PLA

We added 30 ng of the biotinylated capture antibody to streptavidin-coated PCR tubes (Roche Diagnostics) in a total volume of 50 µL and incubated the tubes for 1 h at 37 °C. After washes with PBS containing 0.5 mL/L Tween 20 (Labkemi), the tubes were blocked with an appropriate blocking solution for 1 h at 37 °C. The tubes used for PPV detection were blocked with PBS containing 100 mL/L sheep serum (SVA) and 100 g/L sucrose (Merck). For Lawsonia, the sheep serum was replaced by fetal bovine serum. Tubes were washed with PBS containing 0.5 mL/L Tween, and the sample was added in a volume of 50 µL and incubated for 1 h at 37 °C. Unbound material was removed by washes. A mixture of the proximity probes (24 pmol/L) was then added and incubated for 1 h at 37 °C. The washing step was repeated, 45 µL of the combined ligation and PCR mixture was added, and the real-time PCR was performed according the protocol described for the solution-phase assay. All washes were performed in 3 steps in 200-µL volumes.

QUANTIFICATION OF PPV AND L. intracellularis IN BIOLOGICAL SAMPLES BY PLA

To determine the sensitivity of the test systems, we serially diluted viral and bacterial cell cultures with a known bacterial or viral TCID50 titer in a negative field sample (representative amplification curves are shown in Fig. 1 in the Data Supplement that accompanies the online version of this article available at http://www.clinchem.org/content/vol52/issue6). The cutoffs were defined as values 2 SD above the background signal. To quantify the amount of infectious organisms in the field samples, we compared the signals with a standard dilution series. Before used in the different test systems, the fetal materials to be tested for PPV were diluted 1:30 (30 µL in 870 µL of PBS) and the Lawsonia fecal samples 1:100 (10 µL in 990 µL of PBS).

qPCR of PPV

qPCR analyses of PPV-infected samples were performed with primers used for traditional PCR, as described by Kim and Chae (22). Samples were denatured for 10 min at 95 °C and subsequently analyzed by qPCR. We analyzed 1 µL of each sample in a total volume of 50 µL containing 1× PCR buffer [50 mM KCl; 10 mM Tris-HCl, pH 8.3 (Invitrogen); 3.15 mM MgCl2 (Invitrogen)], 200 µM each of the deoxynucleoside triphosphates, 100 nM each of the primers (forward, 5'-AGCAACACGAATTAGGCCAG-3'; reverse, 5'-GGTCACCATGGATATTCA-3'; Biomers.net), 0.15× SYBR® Green I nucleic acid stain (FMC Bioproducts), and 1.5 U of Platinum Taq Polymerase (Invitrogen). The reaction was cycled 45 times (95 °C for 15 s, 55 °C for 1 min) with an initial denaturing step of 95 °C for 2 min. Organ samples were diluted 30-fold in PBS before analysis.
qPCR of *L. intracellularis*
Samples were denatured for 10 min at 95 °C, diluted 100-fold in PBS, and subsequently analyzed for *L. intracellularis* by a TaqMan assay as described by Lindecrona et al. (23), except that the intercalator dye SYBR Green I was used as a reporter in the assay at a 0.15× concentration.

**ERROR ANALYSIS**
We tested samples in duplicate or triplicate. When a sample was tested in duplicate, the test was repeated. PLA analysis of the standard dilution series of PPV and *L. intracellularis* was performed in triplicate on one occasion. The data presented in Fig. 4A and Fig. 5A represent the mean values at each detection point. The CVs were determined, and the CVs of copy numbers 1.3 and 20 000 are presented in the figure legends. PLA analyses of the PPV fetal samples were performed twice, once as duplicate and once as singleton samples, and the SDs for each sample were calculated. PLA analyses of the *Lawsonia* fecal samples were performed once in triplicate, and the SDs for each sample were calculated.

**Results**

**FUNCTIONALITY TEST OF BIOTINYLATED mAbs**

mAbs previously shown to work well in capture ELISA (A. Nordengrahn, unpublished data) were biotinylated and then tested in the ELISA to examine the extent of binding and biotinylation. The biotinylated mAbs (0.1–0.2 mg/L) could be diluted up to 10- to 12 000-fold and still provide a positive signal (data not shown).

**CHOICE OF REAGENTS, PLA vs ELISA**

It is important to select mAbs (single or used in combination) with high affinity and specificity for use in PLA reactions (8, 24). To pick the best mAb candidates for the *Lawsonia* PLA, we used existing information from the selection of mAbs for the in-house capture ELISA (Svanova Biotech). The mAbs yielding the best results in ELISA were also the optimal choice for PLA, providing the highest specific signal to background in both test systems (Fig. 3).

**COMBINING ANTIBODIES FOR HIGHER SENSITIVITY AND SPECIFICITY**

PLAs in which several antibodies are combined can increase both the specificity and sensitivity of detection. The specificity of the assay increases as the number of specific epitopes that have to be recognized on the target is increased, reducing the risk of cross-reactive detection. The improbability of ligation of the proximity probes in the absence of the specific target ensures a low background and, thus, high assay sensitivity. As shown in Fig. 3A, a *L. intracellularis* culture was diluted in PBS and...
analyzed by PLA using 1, 2, or 3 different mAbs. PLAs with either 1 or 2 antibodies were carried out in a homogeneous-phase assay, and PLAs with 3 antibodies were performed with 1 capture antibody and 2 different antibodies for proximity ligation-based detection. As expected, assay sensitivity was enhanced when 2 or more mAbs were used. The increase in sensitivity in the solid-phase assay with 3 mAbs is probably partly attributable to the washing steps, which reduce background by removing excess reagents before ligation and qPCR amplification of the ligated proximity probes.

**DETECTION OF PPV**

We serially diluted in vitro-cultured PPV of a known TCID$_{50}$ titer in a negative fetal tissue sample and analyzed the dilutions once in triplicate by solid-phase PLA to determine assay sensitivity. Results were compared with those obtained by ELISA and by qPCR of a genomic DNA sequence of the pathogen. As shown in Fig. 4A, 1 copy or just a few copies of viral particles could be detected in a volume of 50 µL by PLA, far below the detection limit of the capture ELISA used for comparison of protein detection. The detection limit was equivalent to that of qPCR. Samples from 20 fetuses were analyzed twice, once in duplicate and once as singleton samples, by solid-phase PLA, qPCR (Fig. 4B), and homogeneous-phase PLA (data not shown). The samples had previously been classified as either positive or negative for PPV by several techniques (17, 18). Analysis of the viral concentration was performed by solid-phase PLA because the limit of detection of the solid-phase PPV assay was lower than that of the homogeneous-phase PPV PLA and because the samples contained substances that inhibited the PCR step. The PLA correctly identified all samples as either positive or negative, with the results from the previously performed HA test used for the comparison. The background noise of the negative samples was significantly lower than the signal observed in samples expected to contain 1 viral particle, which is the cutoff of the assay. The number of virus particles in the positive samples varied from 10 000 to 10 000 000 copies per 50-µL sample. The quantification of virus particles showed a good correlation with the other detection methods.

**DETECTION OF L. intracellularis**

To investigate the sensitivity of the test, we serially diluted a Lawsonia bacterial culture of known concentration in a negative sample. The dilutions were tested once in triplicate; the mean value for each dilution from this evaluation are presented in Fig. 5A. One or just a few bacteria were readily detected in 1-µL samples by the homogeneous-phase PLA assay. By contrast, the detection limit of the capture ELISA was much higher, showing that proximity ligation is superior to the ELISA for detecting low numbers of Lawsonia bacteria. The detection limit of the qPCR for detecting a genomic sequence of the bacteria was similar to that of the PLA test. The homogeneous-phase PLA assay gave results similar those obtained with the solid-phase assay with high sensitivity and specificity; it was therefore chosen for presentation of the results.

We analyzed 20 fecal samples in triplicate with the homogeneous-phase PLA and qPCR (Fig. 5B). The samples previously had been classified as either positive or negative by an in-house capture ELISA (Svanova Biotech), but the numbers of bacteria were not calculated. Numbers of bacteria varied from 1000 up to almost 100 000 in the positive samples and <1 bacterium in the negative samples. The cutoff was set to 1 bacterium per sample. As shown in Fig. 5B, all samples except 3 consistently scored as either positive or negative in both PLA and qPCR. The 3 discordant samples were weakly positive in both PLA and qPCR but negative in the other assay. As with PPV, the quantification of bacteria showed a fairly good correlation with the results obtained from qPCR, although the calculated number of bacteria differed by a factor of 10.

**Discussion**

Our results demonstrate that the assays provide very sensitive detection, allowing direct measurement of viruses and bacteria over 3–4 orders of magnitude in complex biological samples such as fetal tissue and fecal material, with no need for sample preparation. The proximity ligation reaction can be performed as a convenient homogeneous assay, with no need for washes or separation steps. The homogeneous assay format allows protein detection in 1-µL sample aliquots while reducing reagent consumption by 1000-fold compared with regular ELISAs (8). Alternatively, proximity ligation can be carried out as a solid-phase assay, similar to the capture ELISA format, allowing very sensitive analysis of complex tissue samples because excess reagents and any substances interfering with the assay can be removed by washes (7). The assay is equivalent in sensitivity and precision to qPCR, and it is more sensitive than the 2 capture ELISAs used in this study. The low nonspecific background in combination with PCR amplification makes the PLA assay highly sensitive, enabling detection of single or just a few microbe particles. The use of very low concentrations of antibody reagents keeps the assay background noise very low, reducing the chance for proximity in the absence of targets. The high concentration of the connector oligonucleotide may also contribute to the low background by hybridizing to all proximity probes that have not bound to target molecules, thereby inhibiting the target-independent formation of new ligation substrates (7). The use of 3 rather than 2 mAbs for detection further enhanced the specificity and sensitivity of the PLA for the bacterium L. intracellularis. Assay quantification was quite accurate in samples with high microbe copy numbers, but it became imprecise when the number of microbes approached unity. This variation was expected because of sampling errors attributable to the stochastic Poisson distribution of low microbe copy numbers (25, 26). PLA can clearly distinguish between positive and negative cases in field...
Fig. 4. Detection of PPV.
(A), measurement of dilutions of PPV by 3 different methods: solid-phase PLA (■), qPCR of viral genomic DNA (○), and ELISA (□). The viral samples were diluted in a negative tissue sample. The x axis displays the mean values of the total number of viral infectious units (TCID$_{50}$) present in 50-, 1-, and 100-μL samples for solid-phase PLA, qPCR, and ELISA, respectively. The left-hand y axis displays the signal-to-noise ratio for the PLA and qPCR, and the right-hand y axis shows the absorbance at 450 nm for the ELISA. Each sample was run in triplicate. The CVs for the 1.3-copy number data points were 76% and 64% for PLA and qPCR, respectively, and the CVs for the 20,000-copy number data points were 1% and 8% for PLA and qPCR, respectively. The large variation in the 1.3-copy number sample is expected to result from the stochastic distribution of low microbe copy numbers between wells. (B), analysis of lysed pig fetuses by PLA (■), qPCR (○), and HA test (□); samples 1–10 were positive and samples 11–20 were negative for the PPV virus. Triplicate measurements [with SDs (error bars)] of the number of PPV infectious units per 1 μL estimated by PLA and qPCR analysis are indicated on the left-hand y axis, and the HA results are indicated along the right-hand y axis.
Fig. 5. Measurement of the bacterium *L. intracellularis*.

(A), dilutions of the bacterium were measured by 3 different methods: solid-phase PLA ( ), qPCR of viral genomic DNA ( ), and ELISA ( ). The bacterial samples were diluted in a negative feces sample and tested in triplicate. The x axis displays the mean total number of bacteria present in 1-μL samples for the homogeneous-phase PLA and qPCR and in 100-μL samples for ELISA. The left-hand y axis displays signal-to-noise ratios for PLA and qPCR analyses, and the right-hand y axis displays the absorbance at 450 nm for the ELISA. Each sample was assayed in triplicate. The CVs for the 1,3-copy number data points were 67% and 80% for PLA and qPCR, respectively, and the CVs for the 20,000-copy number data points were 18% for both the PLA and qPCR. (B), 20 feces samples, positive (samples 1–10) or negative (samples 11–20) for *L. intracellularis*, were analyzed by PLA, qPCR, and ELISA. Triplicate measurements [with SDs (error bars)] of the number of bacteria in each 1-μL feces sample were obtained by PLA and qPCR (left-hand y axis) and by ELISA (right-hand y axis).
samples from animals with the relevant diseases. In most cases, the background noise of the corresponding negative samples was lower than detection signals from assays of single infectious particles. Quantification of the bacterial or viral particles in the samples showed good correlation with protein detection methods such as the HA test and capture ELISA.

In conclusion, PLA is a simple and reliable tool for detection of viral or bacterial infection. Because only 2 additions of reagents and no washes are needed, this homogeneous-phase assay is well suited for automated analysis of hundreds of samples in parallel. Simultaneous screening of several different infectious agents may be possible with multiplexed proximity ligation reactions, a very important advantage in many clinical applications.

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