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Correspondence and
requests for materials
should be addressed to
P.V. (p.veys@cra.
wallonie.be)

* These authors
contributed equally to
this work.

Determination of the ruminant origin of bone particles using fluorescence in situ hybridization (FISH)

M. C. Lecrenier[1,2]*, Q. Ledoux[1]*, G. Berben[1], O. Fumière[1], C. Saegerman[2], V. Baeten[1] & P. Veys[1]

[1]Walloon Agricultural Research Centre CRA-W, Chaussée de Namur, 24, B-5030 Gembloux, Belgium, [2]Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20, B-42 Sart-Tilman, B-4000 Liege, Belgium.

Molecular biology techniques such as PCR constitute powerful tools for the determination of the taxonomic origin of bones. DNA degradation and contamination by exogenous DNA, however, jeopardise bone identification. Despite the vast array of techniques used to decontaminate bone fragments, the isolation and determination of bone DNA content are still problematic. Within the framework of the eradication of transmissible spongiform encephalopathies (including BSE, commonly known as "mad cow disease"), a fluorescence in situ hybridization (FISH) protocol was developed. Results from the described study showed that this method can be applied directly to bones without a demineralisation step and that it allows the identification of bovine and ruminant bones even after severe processing. The results also showed that the method is independent of exogenous contamination and that it is therefore entirely appropriate for this application.

In recent years, interest in DNA-based identification has increased greatly in many scientific disciplines, not only in archaeology[1] and forensic medicine[2], but also in feed and food safety[3]. Polymerase chain reaction (PCR) is traditionally used for this purpose.

However, PCR has its limitations in case of the degradation of DNA[4] and exogenous contamination[5]. Another important limitation is that, in its classical format, PCR only gives an overview of the DNA content extracted from an analysed matrix, regardless of the cellular origin (i.e., leucocyte, osteocyte or myocyte). Therefore, the complexity of a material submitted to analysis may create misinterpretation.

Within the framework of the eradication of transmissible spongiform encephalopathies (TSE), the detection and identification of processed animal proteins (PAPs) in compound feed is one of the key challenges in ensuring the highest level of food safety[6]. The prohibition of the use of animal by-products depends on their type, their species origin and the feed's destination. Currently, the detection of PAPs is based on two methods, light microscopy and PCR, which ensure the fitness of feed for its intended purpose[7]. Sometimes, however, the information provided by these methods cannot be combined[8]. The recent re-authorisation of non-ruminant PAPs in aquafeed[9,10] requires additional analysis to resolve such cases, and the determination of the ruminant origin of individual animal particles based on the identification of its remnant endogenous DNA is crucial.

Fluorescence in situ hybridization (FISH) is a cytogenetic technique used to detect and localise the presence of specific DNA sequences[11]. In situ hybridization (ISH) was initially and independently developed by Pardue and Gall[12] and by John et al.[13] within the context of intact cytological preparation. The DNA probes were radio-labelled and the detection was carried out by autoradiography. In the FISH procedure, radioactivity was replaced with fluorescence[14]. FISH protocols are now based on the specific hybridization of a fluorescently labelled probe with its complementary target sequence[15]. Hybrids formed between the probes and their DNA targets are detected using fluorescence microscopy.

FISH is widely used for several applications in clinical diagnosis and research[16], as well as for the detection and localisation of genomic aberrations, gene mapping[17], the localisation of gene expression[18] and microbiological diagnosis[15]. Microbiological diagnosis is used in food safety to detect microbial contaminants[19].

The study reported in this paper proposes a new detection method for feed analysis based on the specificity and flexibility of DNA labelling combined with the sensitivity of microscopy. It describes the development of fluorescently labelled probes for the specific identification of bovine and ruminant bones and an original FISH protocol adapted for bone particles that have not undergone the usual demineralisation step. The study also

demonstrates the suitability of this technique for the analysis of highly degraded bone particles regardless of the exogenous DNA environment.

Results

Experimental design. The method was developed using bone particles as these represent the usual microscopic evidence of PAPs in feed enabling the identification of 2 clusters of origin, terrestrial and fish, without further taxonomic characterisation. In addition, DNA is generally well preserved within the bone matrix, even in fossils[20] or after a rendering process.

Five home-made autoclaved bone meals from bovine (BBM), porcine (PBM), ovine (OBM), chicken (CBM) and salmon (SBM) origins were prepared for the study.

Two carcass meals (CarcMs) obtained from a pilot plant were also used: a bovine meal (BCarcM) and a porcine meal (PCarcM). These meals, which contained a high number of bones, had previously been used in the STRATFEED project[21]. These CarcMs, treated at 133°C and at an absolute pressure of 3 bars for 20 minutes, were chosen in line with the sterilisation conditions established by European legislation[22].

A negative control was prepared for checking the absence of FISH marking: a fraction of prepared BBM was reduced to ash in a muffle furnace until there was complete mineralisation of the organic compounds (including the nucleic acids), but with the morphological bone structures still preserved.

Commercial milk powder was used for the preparation of two contaminated (cont-) bone meals (cont-BBM and cont-PBM) in order to obtain a severe exogenous contamination.

The choice of probe sequences for FISH was made on the basis of specificity, sensitivity and ease of tissue permeation[15].

For the bovine probe, a bovine mitochondrial sequence of 59 bases (5′-GGCGACATTGGTTTCATTTTAGCAATAGCATGGTTCCT-AACAAATCTCAATACCTGAGA-3′) was selected from a previously described region used in PCR for its specificity and high number of copies[23].

For the ruminant probe, a ruminant nuclear sequence of 59 bases (5′-CCAGCATCAGAGTCTTTTCCAAATGAGTCAACTCTTCG-CATGAGGTGGCCAAAGTACTG-3′) was chosen using the same criteria (Fumière *et al.* (2012). [Validation study of a real-time PCR method developed by TNO Triskelion bv for the detection of ruminant DNA in feedingstuffs] Unpublished raw data).

During microscopic analysis, bone particles were selected in brightfield, with only those particles clearly identifiable as bones (i.e., with visible lacunae and canaliculi) being chosen.

Bovine probe validation. The bovine probe was used on test portions of BBM, PBM and ashed BBM. Sixteen repetitions of analyses for each home-made bone meal (BBM and PBM) and four repetitions for the ashed BBM were performed. For each repetition, 20 bone particles per slide were examined.

Comparative observations of BBM, PBM and ashed BBM were used to check the absence or presence of a positive signal. Bone lacunae were visualised by brightfield microscopy (Figures 1a, 1b and 1c). Using epifluorescence, well-delineated fluorescent spots were observed on the bovine bone particles (Figure 1d), but these were mostly absent from the porcine particles and were completely absent from the ashed bovine bone particles (Figures 1e and 1f). Composite images showed that in the bovine bones, the light spots were located within the lacunae (Figure 1g) and corresponded to an internal structure in the lacunae, which might have been linked to osteocyte remnants (Figure 2). No other light spot markings were detected elsewhere in the bovine bones (i.e., in the surrounding matrix or canaliculi). These bright fluorescent spots differed from other fuzzy fluorescent areas unrelated to a lacuna and from the
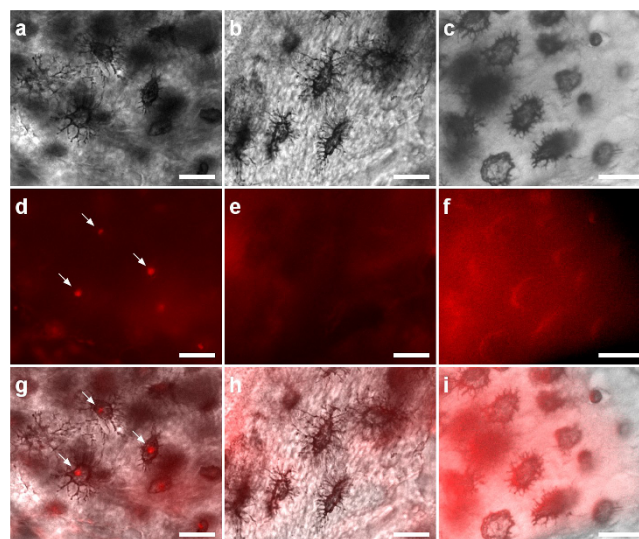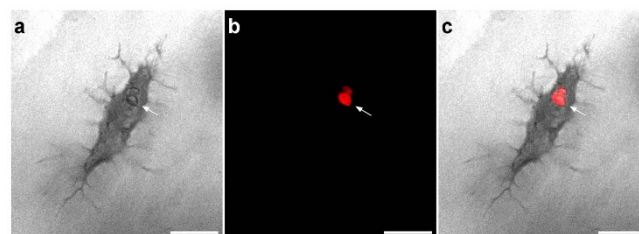

Figure 1 | Micrographs of processed bones hybridized using an ATTO 565-labelled bovine DNA probe. (a–c): Bone particle in brightfield of bovine (a), porcine (b) and ashed bovine origin (c). (d–f): Corresponding images in epifluorescence for bovine (d), porcine (e) and ashed bovine (f) bone. Composite images of the brightfield and epifluorescence images shown for bovine (g), porcine (h) and ashed bovine (i) bone. The arrows point to in situ hybridization spots. Scale bars = 20 μm.

diffuse background fluorescence that was equally present in all bone types (Figures 1g, 1h and 1i).

A positive signal was defined as a co-localisation of a fluorescent spot and a lacuna. The positive staining criterion was consecutively defined with different thresholds in terms of the number of positive signals. At thresholds of 1, 3 or 10 co-localisations, a particle was declared to be positively stained if the respective minimum numbers of lacunae were co-localised with a well-defined fluorescent spot.

The results of the repetitions are summarised in Supplementary Table 1. The method showed that the ashed bovine bones, used as a negative control, were never tagged with the probe. To avoid the misinterpretation of an accidental co-localisation of a hybrid and a lacuna, the threshold was arbitrarily fixed at 3 co-localisations. The threshold of 3 co-localisations corresponded to a mean percentage of 1.5% of the particle lacunae (the average number of lacunae of the particles under analysis was approximately 200). The rate of correctly marked bovine bone particles reached 92.5%, whereas only 6.2% of porcine bone particles were erroneously tagged. This rate of false positive detection for porcine bones was in the same range as the false negative responses for bovine bones.

Figure 2 | Micrographs of bovine processed bone hybridized using an ATTO 565-labelled bovine DNA probe. Bovine bone particle in brightfield (a) and its corresponding image in epifluorescence (b). (c) shows the composite image of the brightfield and epifluorescence images. The arrows point to an internal structure within the lacuna, which corresponds to the hybridization spot. Scale bars = 10 μm.

To evaluate the repeatability of the method, the percentage of particles with a positive staining was analysed per slide, resulting in a boxplot (Supplementary Figure 1), which revealed good data repeatability. The plot also showed that the percentage of positive staining in BBM was significantly higher than in PBM (Wilcoxon rank-sum test, $P < 0.0001$).

Ruminant probe validation. Based on the results obtained from the bovine probe, a second series of analyses were performed on a broader taxonomic target designed for ruminant identification. The microscopic records were comparable in all ways to those shown with the bovine probe. The results of the FISH analysis with the ruminant probe are summarised in Supplementary Table 2, Supplementary Table 3 and Supplementary Table 4.

The analyses were initially performed and repeated four times for BBM, OBM, PBM, CBM and SBM (Supplementary Table 2). Forty particles per slide were examined in this case in order to increase the data per slide. The reduction of the slide replicates was justified by the good repeatability of the method, as demonstrated by the bovine probe.

This time, however, in order to determine whether the specificity could be improved by a higher threshold, a second threshold of 10 co-localisations was also applied. The salmon bone particles were never tagged whatever the threshold. At the threshold of 3 co-localisations, the rate of correctly marked bone particles reached 97.5% and 89.4% for those of bovine and ovine origin, respectively. Only 4.8% of the porcine bone particles and 0.6% of the chicken bone particles were erroneously tagged. With a threshold of 10 co-localisations, the rate of true positive results for the same bone particles fell slightly to 87.5% for the bovine bone particles and to 83.1% for the ovine ones but the rate of false positive results for the porcine bone particles also fell to 1.2%, thus improving the specificity of the method for porcine bones. The rate of positive staining for the chicken bone particles was shown to be stable. The number of false positive results was therefore greatly limited by this threshold.

In order to determine whether exogenous bovine DNA could contaminate bone lacunae and therefore possibly affect the method, two replicates of each material that had been spiked with milk powder (cont-BBM and cont-PBM) were analysed with the ruminant probe (Supplementary Table 3). A total of 80 particles were observed per bone type. With the threshold of 3 co-localisations, 98.8% of the particles in cont-BBM were positively stained, as opposed to 3.8% in cont-PBM. With the threshold of 10 co-localisations, 95% of the particles in cont-BBM were positively stained, as opposed to 1.3% in cont-PBM. A comparison of these results with those obtained for BBM and PBM without contamination (Supplementary Table 2) showed that the contamination had no significant effect (Fisher's exact test, $P > 0.05$) on the results, whatever the threshold or bone species. The strength of the new method was clearly apparent here in that, when analysing the cont-PBM sample using real-time PCR with the ruminant PCR assay[24], it produced a very early signal (data not shown), indicating the presence of the bovine DNA deriving from milk powder.

Finally, two replicates of BCarcM and PCarcM materials were analysed with the ruminant probe in order to determine the applicability of the method to industrial meals. With these materials, as illustrated with BCarcM, the bone surface was opaque in brightfield (Supplementary Figure 2) making bone particles unidentifiable or reducing the total number of lacunae per bone particle that could be interpreted. Preliminary tests confirmed that the threshold of 3 or 10 co-localisations could not be applied. Particle analysis was therefore performed only with the threshold of 1 co-localisation. Under this condition, 52.9% of the particles in BCarcM were positively tagged, but no PCarcM particles were tagged (Supplementary Table 4).

Discussion

The use of a FISH method opens up new possibilities for the determination of the bovine and ruminant origin of bones. Due to the structure of bone and its processing, however, specific care is required.

DNA is a molecule with the capacity to withstand many severe processes. Some conditions, however, such as PAPs processing methods, microorganism degradation and environmental alterations, will result in DNA fragmentation, even though DNA in bones is protected to some extent by mineral sorption[25,26]. In these cases, the length of the chosen target has to be slightly smaller than the average size of a DNA fragment length[23], while retaining a good specificity[27]. The probe length was therefore adapted and fixed to 59 bases.

As the first aim of this method development was detecting ruminant bones, bovine and ruminant probes were synthetized. A porcine probe was also developed to complete our results (data not shown). Until now and despite an evidence of hybridization in the lacunae of bovine and porcine bones particles, the washing process didn't lead to a specific hybridization with the porcine probe. Other developments are in progress.

Direct fluorescent labelling was preferred to indirect labelling. Although indirect labelling can generate a stronger fluorescence signal, it has the disadvantage of requiring additional incubation steps in order to bind the antibody and avidin reagents. The introduction of fluorescent antibodies can also increase the background fluorescence because of the non-specific binding of the antibodies and avidin proteins to extraneous cellular material on the microscope slide, and the slide surface itself[28].

The selection of the fluorochrome has to take account of the bone's natural autofluorescence, due mainly to collagen[29], with maximum emission intensity under ultraviolet light excitation. Early on in the development of the method, Alexa Fluor 488 ($\lambda_{excitation}$ max: 495 nm, $\lambda_{emission}$ max: 519 nm) had been chosen, but it was not possible to distinguish between the probe signal and the bone autofluorescence. ATTO 565 was therefore used for labelling in order to get free from the significant background autofluorescence of the bone particles.

Due to the properties of the bone fragments (size, mineral matrix), standard FISH procedures[30] were not applicable. A new FISH protocol was therefore developed.

Denaturation, hybridization and washing steps were performed in 500 µl micro test tubes, rather than on slides as is usually the case in FISH protocols.

Bone meals were pre-treated with petroleum ether in order to remove the fat from the particles. This pre-treatment was efficient except in the case of CarcMs where an opaque layer of material covered most of the bone surfaces and often made lacunae visualisation impossible in brightfield (see transparent arrow in Supplementary Figure 2). Under these conditions, co-localisation is more difficult to determine. An attempt to characterise this layer using near infrared spectroscopy was made. The study and comparison of spectra for the ashed BBM, BBM and BCarcM showed an increase in fat content in BCarcM. Other pre-treatments using common fat solvents (heptane, hexane, ethanol and methanol/chloroform), oxygen peroxide, ammoniac solution and even enzymatic detergent solution were tested, without satisfactory results for these bone meals.

Each probe was evaluated for its ability to effectively identify its target by calculating the sensitivity and specificity. The calculations were performed for each defined threshold level of co-localisations per particle.

In initial experiments using the bovine probe, the threshold was arbitrarily set at 3 co-localisations, obtaining high levels of both sensitivity (93%) and specificity (94%).

At the same threshold, the ruminant probe revealed a sensitivity of 98% for the detection of bovine bone particles and 90% for ovine

bone particles. Specificities of 96% and 99% were calculated for porcine and chicken bone particles, respectively. At a threshold of 10 co-localisations, specificity reached 99% for the porcine bone particles, whereas for the chicken bone particles, it remained at 99%. As expected, sensitivity fell slightly to 88% and 83% for the bovine and ovine bone particles, respectively. For the purposes of avoiding false positive results, however, this sensitivity level is acceptable. The probe always showed a specificity of 100% for the salmon bone particles at both thresholds.

False positive results, even though relatively infrequent, will need to be eliminated in future studies. One possible source of explanation for these false positives results may be non-specific hybridizations of the probe. Development of a single molecule FISH (smFISH) assay using many probes to target different regions of the ruminant genome could increase this specificity up to 100% because mis-bound hybrids are unlikely to co-localise[31].

The percentage of positive staining in the cont-PBM at the threshold of 3 co-localisations (Supplementary Table 3) was similar to that for the non-contaminated PBM (Supplementary Table 2). The cont-BBM was used as a positive control to confirm that the probe's hybridization with bone DNA was still possible, even in the presence of milk DNA. As the cont-bone meals delivered a percentage of positively hybridized bones close to that observed in the non-contaminated meals, it was concluded that lacunae were unlikely to be contaminated by exogenous DNA and that the positive signals located within the lacunae resulted from hybridizations between the probe and the endogenous DNA. This represents a great advantage for the new method compared with PCR, as it excludes the possibility of false positive results due to exogenous DNA contamination.

For CarcMs, sensitivity fell to 52.9%. This was probably partly due to the severe conditions applied to the test material during industrial processing, resulting in considerable DNA degradation. Using epifluorescence, however, many well-delineated fluorescent spots were visible, suggesting that hybridization had successfully occurred. These spots could not be considered as a positive targeting, however, because the layer of opaque material covering the bone surface made co-localisation impossible. As described in a previous ring trial report using that same BCarcM[32], bone particles are more difficult to identify in this BCarcM than in other bone meals produced following normal EU rendering procedures. Efforts are now being focused on adjusting the protocol for commercial meals by developing more efficient particle pre-treatment and analyses of recent batches of CarcMs that better reflect the real commercial meals.

Conclusion

The procedure developed in the present study is particularly interesting for the detection of PAPs in feed within the framework of the eradication of TSE, especially during the process of the partial lifting of the ban on the use in feed of category 3 (lower risk) animal by-products. The first official method, light microscopy, identifies particles on the basis of typical and morphologically identifiable characteristics such as muscle fibre, cartilage, terrestrial bones, horn, hair, bristles, feathers, egg shells, fish bones and scales. With regard to processed bones, microscopy is able to distinguish terrestrial bones from fish bones, but is unable to determine lower taxa. The second official method, PCR, is able to detect and identify the presence of animal DNA in feed. Nevertheless, in a few cases, it is not possible to interpret the results. In compound feed for fish, for example, if animal particles of terrestrial origin are microscopically detected and if a positive reaction is obtained with the official ruminant probe by PCR, it is currently impossible to determine whether the feed contains PAPs of porcine origin and dairy products (both authorised in aquaculture) or PAPs of bovine origin (prohibited in aquaculture) and porcine blood meal (authorised). The new FISH method, using a combination of light microscopy and PCR information is currently the best option for addressing such problematic cases. It is for this

reason that we chose to test a ruminant probe on CarcMs as part of this study.

The taxonomic identification of bone origin is also important in other fields, such as archaeology and forensic medicine. The common challenges of this identification process are material contamination with exogenous DNA and molecular damage, which make identification extremely difficult, if not impossible.

This original FISH protocol, based on hybridization in micro test tubes rather than on slides, opens up new possibilities for using FISH applications for a wider range of granular and particulate samples.

Methods

Preparation of bone meals. The five home-made autoclaved bone meals of bovine (BBM), porcine (PBM), ovine (OBM), chicken (CBM) and salmon (SBM) origin were prepared as follows. Fresh bovine bones, porcine bones, ovine bones and a whole chicken were bought from a local butcher's. A whole salmon was purchased from a local supermarket. Fat, meat and connective tissue were trimmed off the bones. The bones were cooked in boiling water (1 h 30 per kg bone weight) and smashed into small pieces with a decontaminated hammer. The bone surfaces were decontaminated with DNA-Erase™ (MP Biomedicals, Illkirch, France) and then oven dried for 72 h at 45°C. BBM, PBM and OBM were ground at 1 mm with a rotor mill (SR200 Retsch®, Haan, Germany) previously decontaminated with DNA Erase™. Another rotor mill (ZM200, Retsch®, Haan, Germany) was used to grind the CBM and SBM at 2 mm. The bone meals thus obtained were then autoclaved without stirring for 20 minutes at 138°C and at an absolute pressure of 3.2 bars. In order to check the presence of remaining DNA even after this drastic process, BBM and PBM were analysed with similar real-time PCR assays[23] using two mitochondrial targets (bovine and pig) with identical characteristics (e.g. amplicon size). The results showed close cycle threshold values: 27 cycles with the bovine assay for BBM and 25 cycles with the pig assay for PBM. Due to the similar efficiencies of both tests, the amount of DNA can be considered to be within the same range.

In order to separate and concentrate the bone particles, CarcMs were sedimented using tetrachlorethylene, following the European reference method for the detection of processed animal particles by microscopy[7].

A negative control was prepared by reducing a fraction of the prepared BBM to ash by placing it in a muffle furnace (L9/11/SKM, Nabertherm GmbH, Lilienthal, Germany) at 500°C for 7 h. The preservation of morphological features was controlled by light microscopy. Absence of DNA was successfully checked using PCR, with all the targets in use at CRA-W: porcine, bovine[23], ruminant[24], ovine, chicken and fish (data not published).

Commercial milk powder was used to prepare two contaminated (cont-) pure species bone meals, cont-BBM and cont-PBM. Bone meal (1 g) was adulterated with milk powder (5 g) by direct spiking, and was manually whisked for 2 min. The contaminated meals were heated in the oven for 1 h at 90°C in order to produce in-depth impregnation of the bones with the milk powder. The contaminated meals were then submitted to the same sedimentation procedure used for the CarcMs.

Design of labelled oligonucleotide probes. The oligonucleotides were synthesised (Eurogentec S.A., Seraing, Belgium) and labelled at the 5′-end of the probe with ATTO 565 (ATTOTEC GmbH, Siegen, Germany). The probes were purified by reverse phase HPLC and dried.

In situ hybridization. Test portions of the meals were pre-treated by five successive washes with petroleum ether (10 ml per 300 mg), oven dried at 50°C and passed through two sieves (500 μm and 250 μm square mesh) to obtain usable fractions ranging from 250–500 μm for microscopic slide preparation.

Denaturation, hybridization and washing were performed in 500 μl micro test tubes containing 5 mg of the pre-treated sieved test portions as follows. First, 100 μl of hybridization buffer (50 mM NaCl) containing the DNA probe at a final concentration of 2 μM was added to each micro test tube. Denaturation was performed in a thermocycler (T3 Thermocycler, Biometra GmbH, Göttingen, Germany) at 95°C for 10 min and then incubated for 3 h at the probe's melting temperature: 65°C or 68°C for the bovine and ruminant probes, respectively. Temperatures were determined using a Tm calculator for oligonucleotides (http://www.promega.com/techserv/tools/biomath/calc11.htm) and the salt-adjusted Tm was selected. At the end of the hybridization process, supernatants were removed and the meals were washed in 2 × SSC buffer (300 mM NaCl, 30 mM sodium citrate) for 5 min, followed by three washing steps in 0.1 × SSC buffer (15 mM NaCl, 1.5 mM sodium citrate) of 10 min each for the bovine probe and 5 min each for the ruminant probe. Post-hybridization washes were performed at the same respective hybridization temperatures. Test portions were briefly rinsed twice with ethanol, transferred to a microscope slide and air-dried for 5 min. Mounting of slides with Norland Optical Adhesive 65® (Norland Products Inc., Cranbury, USA) was performed as described by Veys and Baeten[33], except that the portions of material were poured onto the slide before the resin and the UV curing time was only 10 sec.

Probe concentration, hybridization and washing stringency (buffer and duration) and the drying method were optimised experimentally in order to obtain the best specificity.

Microscopy. Observations were made using epifluorescence microscopy (Axio Imager A1m, Carl Zeiss, Göttingen, Germany) equipped with an Epiplan-Neofluar 20×/0.50 DIC objective and a light-emitting diode (LED) illumination (Colibri.2, Carl Zeiss, Göttingen, Germany). Green light excitation (λ = 530 nm) combined with a Zeiss filter set 15 (excitation BP 546/12, FT 580, emission LP 590) was selected for detecting ATTO 565 ($\lambda_{excitation}$ max: 561 nm, $\lambda_{emission}$ max: 585 nm). Sequential images in brightfield and in fluorescence were recorded in black and white by Axiocam MRc (Carl Zeiss, Göttingen, Germany) using the multidimensional acquisition module of the AxioVision 4.8.2.0 software (Carl Zeiss, Göttingen, Germany). The fluorescent images were then pseudo-coloured in red and merged with the brightfield images in order to obtain composite images.

Statistical analyses. Statistical analyses were performed using Matlab R2007b (The MathWorks Inc, Natick, USA). Before each analysis, data were cross-checked by the scientific staff.

In order to compare the percentage of positive staining obtained in BBM and in PBM with the bovine probe, a statistical analysis was conducted using a two-sample Wilcoxon rank-sum (Mann-Whitney) test. The Fisher's exact test was used to compare the results obtained with the ruminant probe for BBM and PBM without or with contamination. The level of statistical significance for both tests was set at $P < 0.05$.

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Author contributions

M.C.L. and Q.L. designed and performed the experiments. M.C.L., Q.L. and P.V. analysed the data. M.C.L. wrote the main manuscript text and Q.L. prepared the figures. M.C.L., Q.L., G.B., O.F., C.S., V.B. and P.V. discussed the results and their implications and commented on the manuscript at all stages. All authors reviewed the manuscript and gave their final approval to the submitted version.

Additional information