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Visualisation of *Mycobacterium avium* subsp. *paratuberculosis* in cultured cells, infected sheep and human tissue sections using fluorescent *in situ* hybridization (FISH).

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ABSTRACT

We describe the development, testing and specificity of a modified oligonucleotide probe for the specific detection of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in culture and in infected tissue using fluorescent *in situ* hybridisation and confocal microscopy. The detection of *MAP* in both animal and human tissue using our modified probe allows for a more rapid diagnosis of *MAP* infection compared to the more often applied detection methods of culture and PCR and has the potential for quantification of cellular abundance. This approach would enable earlier treatment intervention and therefore the potential for reduced morbidity.

MAP (*Mycobacterium avium* ssp. paratuberculosis) Crohn's disease Johnes disease FISH

MAP is a known cause of granulomatous enteritis in ruminants and cause of Johne's disease in cattle (Whittington et al., 2012) and this chronic enteric pathogen is significantly associated with Crohn's Disease in humans (Feller et al., 2007). Accurate and reliable detection of *MAP* in animal samples remains a challenge. Due to its fastidious and often extremely slow growth rate it is difficult microorganism to culture. However, faecal culture from infected animals remains the gold standard for detection of MAP (de Kruijf et al., 2017). Previous detection in culture, ELISA and PCR, have determined presence but not histological location of *MAP* in these samples and direct microscopy is impaired by cell wall deficient *MAP* forms that fail to stain with conventional methods. The commonly used PCR detection systems have targeted the presence of insertion sequence IS*900* as a specific proxy for *MAP* in a variety of tissue and natural samples (Bannantine et al., 2023). However, there have been numerous revisions to IS*900* PCR-based detection due to discovery of new sequences that have cast doubt or invalidated previous assays. Recently, Bannantine et al. (2023), analysed IS*900* loci from 14 closed *MAP* whole genomes and showed that there were errors in sequences of historical *MAP* genomes as well as in the extensively used PCR primer pair P90 and P91 (see Millar et al.1996). Bannantine et al. showed that the forward primer P90 was in fact missing two nucleotides (GC) at positions −7 and − 8 from the 3′ end and proposed a new PCR primer pair using a corrected P90 primer designated P90Corr. Previously, Romero et al. used a 20-mer derivative of the original (and therefore, incorrect) P90 oligonucleotide (missing the first 7 bp) to detect MAP by FISH in surgical tissue from patients with Crohn's disease (Romero et al., 2005). This original description of the P90-derived FISH-probe also contained an error in that the first nucleotide was a C instead of a G (as per the original P90 primer; Millar et al., 1996, Romero et al., 2005). Here we report on the use of the corrected version of the Romero et al. (2005) FISH probe incorporating GC nucleotides at positions −7 and − 8, termed P90CorrProbe (5'-GTTCGGGGCCGTCGCTTAGG-3′).

We show that this new probe is able to detect *MAP* in pure cultures, in sheep tissue known to be infected with *MAP* and visualise presumptive *MAP* infection in human mucosal tissue from a Crohn's patient.

Tissue samples from the terminal ilium of a *MAP*-infected sheep were obtained from the Moredun Institute, Penicuik Scotland. Infection was confirmed from gross pathology reports as well as histological

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investigation. Samples transported in RNAlater were frozen in optimal cutting temperature (OCT) medium (Cell Path Powys) and cryo-sectioned (as described by Arcega et al., 2019, Ross et al., 2022, Ramir et al. 2019). Cultured cells from mycobacterial strains for use in FISH (Table 1), were centrifuged (10,000 x*g*) for 10 min and the cell pellet washed three times in PBS (pH 6.8). Cells were resuspended in 150 μl of PBS, air dried onto slides and heat fixed at 70 °C for one hour.

Human 5 mm colonic biopsies from the ascending colon of an active colonic Crohn's disease patient were obtained by endoscopy and immediately snap frozen in liquid nitrogen. Tissue was cryo-sectioned prior to staining with FISH (Ross et al., 2022, Ramir et al. 2019).

Optimal FISH hybridisation comprised 150 ng of freshly prepared P90CorrProbe mixed with 20 μl of hybridization solution [1% Triton X-100, 2× SSC, denatured sperm DNA (500 μg/ml), 10% dextran sulphate, and 50% deionized formamide], heated to 100 °C and boiled for 10 min, and placed on ice for a further 10 min. The hybridization mixture was then added to the bacterial cells/sheep/human tissue sections and placed inside a hybridization chamber (Affymetrix 645) overnight at 40 °C. Subsequent treatment was as described by Romero et al. (2005).

Slides were viewed with a Leica SP2 system. Two simultaneous confocal channels (Alexa 488 {excitation 493, emission 519} and propidium iodide {excitation 535 emission 615}) were used to collect combined fluorescence images. Separate channels for Alexa 488 and bright field transmission were used to combine fluorescence and phase contrast images. A total of 6 fields per sample were viewed and recorded by two independent observers and representative images shown in Fig. 1. All images were taken at x630 magnification and saved for documentation in JPEG format.

The P90CorrProbe-based FISH assay was tested against different isolates of *MAP* and a range of relevant mycobacterial and non-mycobacterial strains (Zhang et al., 2022; Table 1).

Auramine staining was used as a primary confirmation of the presence of mycobacteria and as a differentiation from non-mycobacterial controls strains (Table 1). The P90CorrProbe-based FISH assay only hybridised to *MAP* isolates (Fig. A, B) with an improved fluorescence intensity when compared to the original P90 probe (data not presented; Romero et al., 2005). P90CorrProbe staining of a sheep terminal ileum sample was able to locate *MAP* within multiple cells within the lamina propria (Fig. 1C) and showed a perinuclear distribution of fluorescent signal, accentuated when counterstained with propidium iodide (Fig. 1D). The pathology of the sample of intestine showed a severe infiltration of inflammatory cells associated with Johne's disease. The inflammatory cells were predominantly macrophages characterised by a large cytoplasm, but areas with eosinophils were also observed. Some of the macrophages contained acid fast organisms. *MAP* was also identified by this assay in the samples from the ascending colon of a male patient with active colonic Crohn's Disease (Fig. 1E, F).

We have tested a new FISH oligonucleotide (P90CorrProbe) for IS900 detection, correcting sequence anomalies identified by this study and those presented by Bannatine et al. (2023). This P90CorrProbe was shown to be specific for *MAP* strains containing IS900 and with improved fluorescence intensity clearly localised to macrophages in tissue from sheep with Johne's Disease to acid-fast bacteria observed during pathological examination and in the lamina propria from the ascending colon of human CD patient. The ability to directly visualise and determine location of MAP in human tissue will be an important step in understanding the potential role of *MAP* role in the aetiology of

Table 1
Testing of P90CorrProbe-based FISH assay for the specific detection of *MAP*.

Identification	Reference strain/origin	Auramine	MAP-FISH
Mycobacterium avium subsp. *paratuberculosis* (MAP) K10	Cattle reference strain (ATCC BAA 968)	+	+
MAP 3737	Bovine strain, R J Chiodini et al. 1993	+	+
MAP Gallop	*MAP* isolate from CD patient. (provided TJ Bull, UK)	+	+
MAP CLIJ644	Cattle with Johne's Disease (Australia; Wynne et al., 2011)	+	+
MAP Linda	*MAP* isolate from CD patient. ATCC 43015	+	+
Mycobacterium species (M2333)	Cattle isolate. (Gift from G. Bolske Sweden: Englund et al., 2002)	+	–
M. avium subsp. *avium* 8559	Chicken (NCTC8559)	+	–
Mycobacterium avium subsp silvaticum 10	Wood pigeon (CIP 103317 ATCC 49884)	+	–
Mycobacterium species (M22850)	Soil (Gift from D Cousins Australia; Cousins et al., 1999)	+	–
M. vaccae	NCIMB 9937/ATCC 15483	+	–
UK vaccine strain BCG1331	Recovered from UK batch of BCG vaccine 2023	+	–
Pseudomonas putida	ATCC 23467	–	–
Bacillus sp.	ATCC 14884	–	–
E. coli DH5	ATCC BAA-3219	–	–
E. coli AIEC	Elliott et al. (2013)	–	–
E. coli non-AEIC	Elliott et al. (2013)	–	–

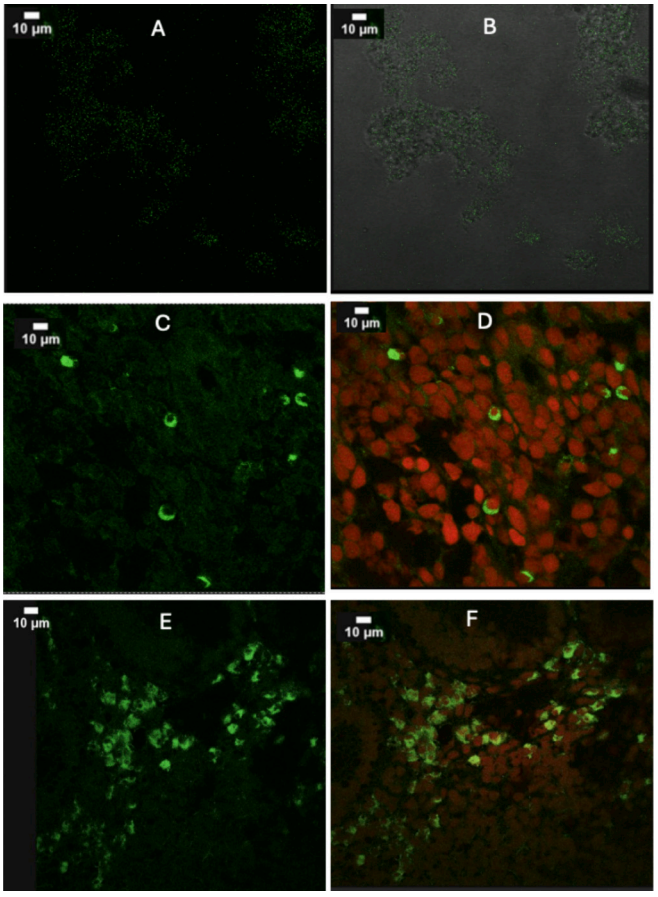

Fig. 1. FISH hybridization of P90CorrProbe to detect *MAP* in pure culture, sheep and human tissue. Fluorescence staining of *MAP* (*Gallop* strain) in pure culture alone (A) and a combined fluorescence and phase contrast image (B). *MAP* in diseased sheep tissue, fluorescence only (C) and combined fluorescence/propidium iodide in order to demonstrate cell nuclei (D). *MAP* in human tissue from the ascending colon of a Crohn's patient (E) showing *MAP* positive cells in the lamina propria with propidium iodide counterstain (F). All images: *MAP* (green) cell nuclei (red) and are at x630 magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Crohn's Disease (Sharp et al., 2018). In addition to this the ability to rapidly detect and localise *MAP* infection in both animal as well as human tissue, with the potential to quantify the cellular abundance, has a huge benefit in terms of early treatment intervention and reduced morbidity.

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Declaration of generative AI in Scientific writing

No artificial intelligence was used in the preparation of this manuscript.

Ethics statement

Full ethical approval was given for the collection and use of human tissue (IRAS 230528). The sheep tissue was ethically sourced by the Moredun Research Institute. Sheep work was carried out in accordance with the UK Animals (scientific procedures) Act 1986 under two Home Office project licenses: PPL60/3900 (Pathogenesis and Control of Mycobacterial Infections) and PPL60/4428 (Studies of Mycobacterial Infection). The proposed work was also scrutinised by Moredun's own ethics committee (Animal Welfare and Ethics Review Board-AWERB).

CRediT authorship contribution statement

Neil Rayment: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Glenn Rhodes:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Barry Hudspith:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Valerie Hughes:** Writing – review & editing, Resources, Conceptualization. **Francesca Chianini:** Writing – review & editing, Resources, Investigation. **Gaurav Agrawal:** Writing – review & editing. **Tim J. Bull:** Writing – review & editing, Resources. **Roger Pickup:** Writing – review & editing, Writing – original draft, Formal analysis. **Jeremy Sanderson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2024.107001.

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