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Culture-independent PCR detection and differentiation of *Mycobacteria spp.* in antemortem respiratory samples from African elephants (*Loxodonta africana*) and rhinoceros (*Ceratotherium simum*, *Diceros bicornis*) in South Africa.

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Abstract: Since certain *Mycobacterium tuberculosis* complex (MTBC) members, like *M. bovis*, are endemic in specific South African wildlife reserves and zoos, cases of clinically important nontuberculous mycobacteria (NTM) in wildlife may be neglected. Additionally, due to the inability of tests to differentiate between host responses to MTBC and NTM, the diagnosis of MTBC may be confounded by the presence of NTMs. This may hinder control efforts. These constraints highlight the need for enhanced rapid detection and differentiation methods for MTBC and NTM, especially in high MTBC burden areas. We evaluated the use of the GeneXpert MTB/RIF Ultra, the Hain CM*direct* V1.0, and novel amplicon sequencing PCRs targeting the mycobacterial *rpoB* and *ku* gene targets, directly on antemortem respiratory samples from known MTBC-infected and NTM culture-positive African elephants (n=26 animals) and rhinoceros (n=23 animals). Our findings suggest that the Ultra is the most sensitive diagnostic test for MTBC DNA detection directly in raw antemortem respiratory specimens and that the *rpoB* PCR is ideal for *Mycobacterium* genus DNA detection and species identification through amplicon sequencing.

Keywords: African elephants; broncholoalveolar lavage; Hain CM*direct* V1.0; *ku* PCR; *Mycobacterium tuberculosis* complex; non-tuberculous mycobacteria; rhinoceros; *rpoB* PCR; trunk wash; Xpert MTB/RIF Ultra.

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1. Introduction

Mycobacteria are a diverse group of microorganisms that can be found in practically every environmental niche [1]. They are divided into two categories: *Mycobacterium tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM) [2]. *Mycobacterium tuberculosis*, *M. bovis*, *M. orygis*, *M. bovis* bacillus Calmette-Guerin (BCG), *M. africanum*, *M. cannettii*, *M. pinnipedii*, *M. caprae*, and *M. microti* are all members of the MTBC. *Mycobacterium bovis* and *M. tuberculosis* are well known for causing chronic infectious disease in people and animals, including livestock and wildlife species [3–6]. Cases of *M. bovis* in different South African (SA) wildlife, including African buffaloes, African lions, African wild dogs, and rhinoceros are sporadically reported. Disease control efforts for buffaloes include test-and-slaughter strategies and quarantine for endangered species [3,5,7–9]. Many high TB burden countries are heavily dependent on animal-related industries such as tourism and agriculture, leading to habitat encroachment and increased opportunities for disease transmission at the animal-human interface. This phenomenon has recently been highlighted by the unexpected discovery of fatal *M. tuberculosis* infection in an African elephant (*Loxodonta africana*) in Kruger National Park (KNP), which is endemic for *M. bovis* [10,11].

Non-tuberculous mycobacteria, also known as environmental mycobacteria, are natural inhabitants of the environment and comprise more than 150 species listed on public bacterial databases (http://www.bacterio.net). Interestingly, more than a third of these species have been implicated in diseases in livestock, wildlife, and humans [12]. Numerous animal and human NTM infections are reported globally, likely due to their ubiquitous presence and opportunistic nature [13,14]. *Mycobacterium avium* complex (MAC) organisms, consisting of *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. sylvaticum*, *M. colombiense*, *M. bouchedurhonense*, *M. timonense*, *M. chimaera*, *M. arosiense*, *M. yongonense*, and *M. marseillense,* are the most well-known opportunistic NTMs reported in animals and humans [15]. In addition, *M. kansasii*, *M. marinum*, and *M. ulcerans* commonly cause opportunistic NTM disease. Infections with *M. kansasii* can cause pathological signs resembling *M. bovis*-associated disease in animals [12,16]. Another NTM, *M. scrofulaceum* has been found in lymph nodes of cattle, buffaloes, farmed deer, swine, wild pigs, patas monkeys, fish, and mice [17]. These organisms can spread indirectly through contaminated environments, as is evident by the isolation of mycobacteria from cattle and mice faeces [17].

In Africa, most NTMs are reported as incidental findings in livestock with gross pathological lesions identified in slaughterhouses, when tissues are submitted for *M. bovis* surveillance [18,19]. There are substantially fewer reports of NTMs isolated from free-ranging wildlife. Therefore, the extent and distribution of NTM infections among African wildlife species is largely unknown. In SA, *M. avium* subspecies *paratuberculosis*, *M. terrae*, *M. nonchromogenicum*, *M. vaccae*/*M. vanbaalenii*, and unidentified species closely related to *M. moriokense* have been isolated from African buffaloes, livestock, and their environments indicating NTMs may be exchanged at environment-animal interfaces [20]. Besides being opportunistic pathogens, some NTM species may colonize the host without development of disease, but prime the host's immune system, confounding immuno-diagnosis of bovine tuberculosis (bTB) due to cross-reactivity to shared antigens [21,22]. Genetic investigations into NTM isolates from samples collected from various wildlife species showed the presence of orthologous genes like

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ESAT-6 and *CFP-10* situated in the *ESX-1* to *ESX-5* regions [2]. The occurrence of positive *M. bovis* immunological test results in these animals suggest that NTMs potentially interfere with bTB diagnostic assays. Since *M. bovis* is endemic in several SA wildlife reserves and zoos, cases of NTM infection in wildlife may be neglected, due to the primary focus being on *M. bovis* diagnosis. However, due to the inability of tests to differentiate host responses towards MTBC and NTMs, due to the interference of NTMs, may hinder control efforts. Unfortunately, correctly identifying a NTM requires mycobacterial culture with a minimum 6-8-week incubation period and further genetic speciation [23]. This constraint highlights the need for enhanced and rapid MTBC and NTM detection and differentiation assays, especially in high *M. bovis* burden areas.

Assays using PCR have been used to rapidly detect and differentiate MTBC and NTM. Recently, the Cepheid Xpert MTB/RIF Ultra assay (Ultra) has been shown to provide rapid detection of MTBC DNA in tissue and respiratory samples collected from infected African buffaloes, African elephants, and rhinoceros [5,6]. The HAIN GenoType CM*direct* VER 1.0 line probe assay (Hain LPA) can also lead to sensitive and specific detection of DNA from *Mycobacterium* genus organisms, MTBC (without differentiation), and the differentiation of more than 20 clinically relevant NTMs directly from human patient specimens [24]. Although *16S* rRNA sequencing of *Mycobacterium* cultures has been used for speciation, there are increasing reports that this technique is sub-optimal [25]. More recent studies, using hundreds of strains of M*ycobacterium* spp. isolates, have demonstrated that combined PCR amplification of highly conserved regions of the *Mycobacterium ku* and *rpoB* genes, using primers specifically designed for the *Mycobacterium* genus, have superior performance compared to *16S* rRNA amplicon sequencing for genus detection and speciation of low-level mixed microbial populations [25–28]. Consequently, these findings have major implications for the field of targeted next generation sequencing directly from clinical samples, especially for the improved surveillance of clinically important *Mycobacteria* spp. Therefore, the aims of this pilot study were to evaluate the use of 1) the Ultra and Hain LPA for rapid MTBC DNA detection, 2) the Hain LPA for NTM DNA detection and species differentiation without required sequencing, and 3) *ku* and *rpoB* amplicon sequencing for *Mycobacterium* genus DNA detection and species differentiation, directly from antemortem respiratory samples. These samples were collected from known MTBC and NTM culture-confirmed positive African elephants and rhinoceros from KNP and a zoo in SA.

2. Results

2.1. Mycobacterial culture results

Respiratory samples from six animals (five elephants and one rhinoceros) were defined as the culture confirmed MTBC positive cohort for this study (Figure 1 and Table 1). Six of eight elephant respiratory samples, three bronchoalveolar lavage fluid (BALF) samples (including one duplicate sample) and three trunk washes from five MTBC infected elephants (free-ranging and zoo), were confirmed to contain viable MTBC through mycobacterial culture and speciation. The remaining two respiratory samples (TW and BALF) did not grow MTBC but rather *M. elephantis* and *M. stomatepiae* were isolated (Table 1). The infected animals included: 1) two zoo elephants (18/85 and 18/177) from which *M. tuberculosis* was cultured from the first animal's (18/85) BALF sample, *M. tuberculosis* and *M. africanum* from the second elephant's (18/177) duplicate BALF samples and *M.*

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elephantis from its TW sample, respectively and 2) three free-ranging KNP elephants (18/527, 18/533 and 18/538) from which *M. bovis* were cultured from each animal's TW sample (Table 1). Even though KNP elephant 18/527 TW sample contained viable culturable *M. bovis*, its BALF sample did not (Table 1). Lastly, *M. bovis* was also cultured from a BALF sample collected from a white rhinoceros (19/46) from KNP (Table 1).

Respiratory samples from nine animals (eight elephants and one rhinoceros) were defined as the culture confirmed NTM positive cohort for this study (Figure 1 and Table 1). Ten respiratory samples (7 TW and 3 BALF) from eight elephants and a single (BALF) sample from one rhinoceros were confirmed to contain viable NTMs through mycobacterial culture (Table 1). *Mycobacterium mantenii* was successfully isolated and characterized from a KNP elephant's (19/460) BALF sample and *M. abscessus* strain from its TW sample. Similarly, *M. interjectum* was isolated and characterized from a KNP elephant's (18/530) BALF sample and *M. avium* complex from its TW sample (Table 1). Non-tuberculous mycobacteria isolated from TW samples collected from the remaining four KNP elephants and one zoo elephant included *M. avium complex strain* (18/173), *M. mageritense* strain (18/532), *M. intracellulare* (18/534 and 18/539), and *M. fortuitum* strain (21/496). Elephant (18/176) and rhinoceros (18/31) BALF samples were culture positive for *M. foliorum* and *M. scrofulaceum strain*, respectively (Table 1).

Additionally, 25 respiratory samples (15 BALF and 10 TW) from 13 elephants and 21 BALF samples from 21 rhinoceros were considered mycobacterial culture negative by the BACTEC 960 MGIT system and defined as the culture negative cohort in this study (Figure 1 and supplementary material).

2.2. Presence of ESAT-6/CFP-10 in all mycobacterial cultures

All MTBC culture isolates from seven respiratory samples (one rhinoceros and five elephants, including one duplicate BALF sample), were PCR-positive for *ESAT*-6 and *CFP*-10 (Table 1). Nine of the 13 NTMs isolated by respiratory sample culture were also PCR-positive for *ESAT*-6 and *CFP*-10 (one rhinoceros and ten African elephants) (Table 1). However, one of the PCR-positive NTM strains (*M. elephantis*) was cultured from a TW sample from an elephant (18/177) with *M. tuberculosis* and *M. africanum* isolated by culture in BALF samples (Table 1).

Within the culture negative cohort, the presence of *ESAT-6* and *CFP-10* was detected in 7 of 46 respiratory sample cultures (13 elephants and 21 rhinoceros; supplementary material). For these *ESAT-6/CFP-10* positive samples, the potential presence of the following *Mycobacterium* species was also identified by PCR amplicon sequencing directly from the respiratory samples: 1) *M. africanum*, 2) *M. bovis*, 3) *M. interjectum*, 4) *M. intracellulare*, 5) *M. avium* complex, and 6) *M. orygis* (supplementary material). Target amplification was confirmed through Sanger sequencing and using NCBI's Basic Local Alignment Search Tool for nucleotide (BLASTn) [29].

2.3. *Nucleic acid amplification test results on raw respiratory samples*

2.3.1. Ultra and Hain LPA for MTBC DNA detection

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The Ultra successfully identified all five infected elephants and the one infected rhinoceros as MTBC infected, which included four positive BALF and three elephant TW samples out of the nine respiratory samples (7/9) (Table 1). The Hain LPA also correctly identified three of the MTBC infected elephants (18/85, 18/527 and 18/533) based on one positive TW sample and two positive BALF samples. The *M. bovis*-positive rhinoceros (19/46) was also identified as MTBC positive by the Hain LPA using its BALF sample (Table 1). Three of the four positive MTBC results on the Hain LPA agreed with the Ultra results, with a discordant result (negative Ultra; MTBC and/or *M. fortuitum* group Identified by Hain LPA) for the BALF sample of the *M. bovis*-positive elephant (18/527). This BALF sample was *ESAT-6/CFP-10* negative and *M. stomatepiae* was isolated (Table 1). The TW sample from the *M. tuberculosis* infected zoo elephant (18/177), based on BALF culture, only contained culturable *M. elephantis*, which was negative on the Ultra, Hain LPA, and *ESAT-6/CFP-10* PCRs (Table 1).

When Ultra and Hain LPA results were compared using a two-tailed z-test, there was a significant difference ($p < 0.00001$) between these two tests for MTBC detection within the confirmed MTBC infected cohort. However, agreement between the Ultra and Hain LPA for MTBC DNA detection from all specimens (regardless of culture outcome) was "substantial" (κ=0.75, 95% CI 0.55 - 0.96: standard error (SE)=0.10) (30). Both the Ultra and Hain LPA identified the same three elephants (18/173, 19/460 and 18/534) as MTBC infected based on one BALF and two TW samples, although culture isolates categorized these individuals in the NTM positive cohort (Table 1). All three of these respiratory samples were also positive for *ESAT-6/CFP-10* by PCR. Similarly, within the culture negative cohort, the Ultra and Hain LPA detected the same four elephants as MTBC infected based on results from three BALF samples and one TW sample (all *ESAT-6/CFP-10* positive) (supplementary material).

Comparison of the Ultra and Hain LPA results to culture detection showed "substantial" agreement between Ultra and culture for MTBC detection (κ=0.61, 95% CI 0.36-0.86, SE=0.13), although lower than agreement between the Ultra and Hain LPA results (κ=0.75). However, there was only "slight" agreement between Hain LPA and culture results (κ=0.23, 95% CI - 0.07 - 0.54: SE=0.16). When the results of Ultra and Hain LPA were combined and compared to culture for MTBC detection, there was only "moderate agreement" (κ=0.58, 95% CI 0.33 - 0.83: SE=0.13).

2.3.2. Hain LPA NTM DNA detection and species differentiation

The Hain LPA identified all eleven respiratory samples from the culture confirmed NTM positive cohort (eight elephants and one rhinoceros), as containing NTM DNA (11/11). Eight samples (8/11) were also positive for *ESAT-6/CFP-10* amplification and three of those included samples from elephants (18/173, 19/460 and 18/534) that were also identified as containing MTBC DNA by Ultra and Hain LPA (Table 1). The Hain LPA detected mixed NTM DNA (>1 NTM strain) within samples from these three KNP elephants, as well as an additional KNP elephant (18/530) and KNP rhinoceros (18/31) within the NTM positive cohort (Table 1). Similarly, within the MTBC infected cohort, the Hain LPA also detected NTM DNA in eight out of nine respiratory samples, with five respiratory samples showing a mixture of NTM DNA (Table 1). Within the culture negative cohort, NTM DNA was detected by Hain LPA in 19 of 46 respiratory samples. These included four samples from four elephants also identified as containing MTBC DNA by both the Ultra and Hain LPA. Three of these four elephant samples, and six

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additional elephant respiratory samples were shown to have a mixture of NTM DNA based on the Hain LPA (supplementary material). When the Hain LPA was compared to culture results for detection of NTM using all respiratory samples, only "fair" agreement was observed (Table 2). However, when identified NTM species by Hain LPA were compared with culture results, the Hain LPA only correctly identified one culture isolate (KNP elephant 19/460, TW, *M. abscessus* strain) (Table 1).

2.3.3. Ku and rpoB amplicon sequencing for Mycobacterium genus detection and speciation

Nine respiratory samples, confirmed to contain MTBC (*M. tuberculosis, M. africanum, M. bovis*) by culture, showed amplification in the *ku* and *rpoB* PCRs, which identified the presence of the *Mycobacterium* genus DNA (Table 1). Upon amplicon sequencing, both PCRs correctly identified the same MTBC species, directly from respiratory samples, as by culture (Table 1). Discordant culture/*ku* PCR results were observed for two elephant (18/177 and 18/527) TW and BALF samples. Elephant (18/177) TW was identified as containing *M. elephantis* (*ESAT-6/CFP-10* positive) by culture and *rpoB* PCR, but as "*M. fortuitum* group" by the *ku* PCR (Table 1). Similarly, elephant (18/527) BALF was identified as containing *M. stomatepiae* (*ESAT-6/CFP-10* negative) by culture and *rpoB* PCR, but "mixed NTMs/*M. smegmatis"* by *ku* PCR (Table 1). When comparing the results from the *ku* and *rpoB* PCRs, there was no significant difference (two-tailed Z test, p=1) between these two tests for *Mycobacterium* genus detection and MTBC species identification within the culture confirmed MTBC infected cohort.

Both the *ku* and *rpoB* PCRs identified the presence of *Mycobacterium* genus DNA in all but one of the eleven respiratory samples from the NTM positive cohort. The TW sample from KNP elephant (18/532) was culture positive for *M. mageritense* strain (*ESAT-6/CFP-10* negative) but identified as *M. fortuitum* group by Hain LPA (Table 1). The *rpoB* amplicon sequences predicted the same species of NTM as culture, but directly from the respiratory samples; the *ku* amplicon sequences identified five of eleven NTM species assigned after culture (Table 1). Although there was no statistical difference (p=1.0) between the two PCRs for *Mycobacterium* genus detection, there was a significant difference in the ability to identify NTMs ($p < 0.02$).

Within the culture negative cohort, the presence of *Mycobacterium* genus was detected by both PCRs in the same 15 elephant respiratory samples (also all HAIN LPA positive). Two Hain LPA positive elephant (18/537 TW and 18/539 BALF) samples were *rpoB* PCR positive, but *ku* PCR negative; in addition, one elephant 18/157 TW sample that was also Hain LPA positive was *ku* PCR positive but *rpoB* PCR negative (supplementary material). Three respiratory samples from different elephants (18/255, 18/534 and 18/536) were shown to have *M. africanum, M. bovis,* and *M. orygis* DNA, respectively, by both the *ku* and *rpoB* PCRs, although these samples were culture negative but all *ESAT-6/CFP-10* PCR positive. These three samples were also positive for MTBC DNA by both the Ultra and Hain LPA (supplementary material).

To evaluate the best test or combination of tests, including Hain LPA for *Mycobacterium* genus detection, agreement analysis was performed (Table 2). For *Mycobacterium* genus detection, "fair" to "moderate" agreement were observed between culture and individual tests (*rpoB-*, *ku* PCR and Hain LPA) as well as for

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combinations of these tests versus culture results (Table 2). “Almost perfect” agreement was reported between individual tests (*rpoB*, *ku* PCRs and Hain LPA) as well as test combinations (Table 2).

For mycobacterial species identification compared to culture, the Hain LPA only correctly identified a single NTM positive sample (as previously mentioned), and it was incapable of differentiating the MTBCs. Compared to culture results, the *rpoB* and *ku* PCRs both correctly identified the same 6/7 MTBC culture positive samples. For all NTM positive samples, the *rpoB* PCR correctly identified 10/13 NTM culture positive samples with 29/46 culture negative samples also negative on the *rpoB* PCR (Table 1 and supplementary material). The *ku* PCR correctly identified 4/13 NTM culture positive samples and 30/46 culture negative samples were also negative on the *ku* PCR (Table 1 and supplementary material). The four NTMs identified by the *ku* PCR were also correctly identified by the *rpoB* PCR (Table 1).

3. Discussion

This pilot study described the successful culture isolation and genetic speciation of *M. tuberculosis*, *M. africanum* and *M. bovis* from antemortem respiratory samples collected from zoo and free-ranging African elephants and rhinoceros. Our recent culture isolation success was largely due to the combined use of conventional MGIT culture with a novel modified version called MGIT-TiKa [23,31] as well as improved isolate speciation through the simultaneous detection and sequencing of three different genetic markers (*16S* rRNA, *rpoB* and *hsp65*). In the zoo elephant (18/85), *M. tuberculosis* had previously been isolated from lung and lymph node tissue samples [5], and in this study, *M. tuberculosis* was also isolated from its antemortem BALF sample (Table 1). Notably, elephant 18/85 BALF samples were collected before it was euthanized for tissue sample collection, removing the possibility of BALF contamination at necropsy. Moreover, in the same zoo, simultaneous culture isolation of both *M. tuberculosis* and *M. africanum* from duplicate BALF samples was found in a single contact elephant 18/177. This was the only MTBC coinfection discovered among the elephants and rhinoceros in this study. Since this elephant was in a zoo environment in SA with known MTBC infections, the results were not that surprising [32]. The effects of MTBC coinfections on disease development are still largely unknown, especially in zoo settings, but certainly warrant further investigations. The successful culture isolation of *M. bovis* from three free-ranging African elephants and one rhinoceros in a *M. bovis* endemic wildlife reserve like KNP were not unexpected, but surprising that it was cultured from paucibacillary antemortem respiratory samples. These findings suggest that possible MTBC shedding by elephants and rhinoceros may occur in zoos and wildlife reserves, however, it must also be noted that the isolation of *M. bovis* from elephant trunk wash samples could also have been due to contamination from other infected hosts, like African buffaloes, shedding into the environment [33].

Isolation of various NTMs by culture from antemortem respiratory samples like BALF and TW was also expected (Table 1), especially with the novel culturing approach used. It is noteworthy that some clinically important NTMs were isolated along with supportive PCR evidence for the presence of ESAT-6 and CFP-10 virulence factors. These included *M. abscessus*, *M. avium* complex strains, *M. interjectum*, *M. fortuitum* and *M. scrofulaceum* (Table 1). However, one limitation was that all these animals were from MTBC endemic areas and

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M. tuberculosis and *M. bovis* may have been present in small amounts below the detection threshold of culture, but detectable by PCR. This could result in positive *ESAT-6/CFP-10* PCR, but culture negative results (Table 1). For many of these NTMs, opportunistic human and animal infections have been reported [2,34–36]. Two cases of atypical mycobacteriosis caused by *M. szulgai* have been reported in zoo elephants as well as disease caused by *M. kansasii* infection in a bontebok herd, which all were positive for antibodies to ESAT-6/CFP-10 [16,37]. These cases highlight the diagnostic challenges around detection of NTMs and differentiation from MTBC infections. The need to differentiate mycobacterial species is becoming increasingly recognized despite the fact that many NTMs may not cause disease, but could prime the host's immune system, subsequently impeding the accurate diagnosis of MTBC infections, especially when using virulence factor proteins as test antigens [38].

An important finding within the culture negative cohort, was the PCR detection and identification of MTBC DNA (*M. bovis*, *M. africanum, M. orygis*) in antemortem samples (supplementary material). Without this result, any positive immunological assay results would have been classified as false-positive, rather than MTBC infected. These findings were supported by *ESAT-6/CFP-10* positive PCR results, combined by the simultaneous species identifications by three separate tests, the *rpoB* PCR, *ku* PCR, and Hain LPA. However, a significant limitation is the inability of PCRs to differentiate between live and dead bacteria, which would be important for evaluating transmission risk. Therefore, it is likely that shedding in MTBC infected elephants and rhinoceros may occur more frequently than reported due to the paucibacillary nature of antemortem respiratory samples and overall sub-optimal sensitivity of culture [39,40].

The predictive ability of three candidate PCRs (Ultra, *rpoB* PCR, *ku* PCR) and one LPA, used directly on antemortem respiratory samples from culture defined (MTBC, NTM and negative) animal cohorts, was assessed to identify the most sensitive technique (Table 1). For MTBC DNA detection, the Ultra qPCR was significantly (p<0.00001) more sensitive than the Hain LPA with samples from the MTBC culture positive cohort. In addition, one advantage was that the Ultra used raw samples as input material whereas the Hain LPA required DNA extraction. Discordant results between tests were reported for elephant 18/537 (*M. bovis* culture positive TW) where the Ultra detected MTBC DNA only in the TW sample, whereas the Hain LPA detected MTBC DNA in the BALF sample (from which *M. stomatepiae* was isolated by culture). These finding highlights the possibility that MTBC may be present in samples from infected animals but may be unculturable due to their paucibacillary nature and sub-optimal sensitivity of culture. A sample's bacterial load may also be reduced during sampling, sample handling and storage. It is also possible that the TW sample reflected overall respiratory load of bacilli and the possibility of not sampling the infected site using BAL. This highlights the importance of testing multiple samples.

Within the NTM culture positive and negative cohorts, MTBC DNA was also simultaneously detected by both the Ultra and Hain LPA with positive amplification of virulence factors for those samples. Again, this may have been dead bacteria or samples with small unculturable amounts of MTBC bacilli. Agreement between the Ultra, Hain LPA, and their combined use versus culture results for all the samples showed that the Ultra was the most sensitive test for MTBC DNA detection. Combining the results from the Ultra and Hain LPA for MTBC DNA

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detection did not improve overall detection, supporting the individual use of Ultra for MTBC DNA detection directly from raw antemortem respiratory samples, which may identify infected animals that are culture negative.

The Hain LPA for NTM DNA detection and species identification identified all samples from the NTM positive cohort as containing NTM DNA (Table 1). Additionally, it also detected NTM DNA in MTBC positive and culture negative cohort samples, which would be important for identifying co-infections. The Hain LPA produced fair agreement compared to culture for NTM detection, although there was no agreement with culture for species identification. This observation may be due to the use of hybridization technology by the HAIN LPA producing unclear subjective results, especially when used directly on raw animal samples or further evidence of the selective pressure introduced by the mycobacterial culture during sample processing [31]. Most samples were identified by Hain LPA as containing DNA from a mixture of NTM species, but cultures were positive for only a single NTM species. This could indicate that the NTM that was successfully cultured most likely outcompeted the rest during incubation or that they were eradicated prior to inoculation during the decontamination process [31].

Through PCR amplification and subsequent amplicon sequencing of the *rpoB* and *ku* gene targets, using *Mycobacterium* specific primers, both PCRs correctly detected and identified all MTBC species directly from antemortem respiratory samples within the culture confirmed MTBC positive cohort. No significant difference (p=1) for genus detection or species identification were reported between these PCRs within the MTBC positive cohort. Similarly, both PCRs detected Mycobacterium genus DNA from all samples in the NTM positive cohort, with no significant difference (p=1), except one TW sample from a KNP elephant. The negative result for both PCRs may have been due to damaged DNA during sample storage or handling since the same DNA sample produced a positive *M. fortuitum* result in the Hain LPA. Otherwise, amplicon sequencing of the rpoB target identified all cultured NTM species correctly with 100% accuracy, directly from respiratory samples, unlike the sequenced *ku* amplicons that only predicted 45% of the cultured species. A significant difference (p<0.02) between the *rpoB* PCR and *ku* PCR were observed for species identification. The success of the *rpoB* PCR for culture isolate prediction is based on a study performed by Adékambi *et al*. (2003), where the authors focused on a 723 bp variable region exhibiting 83.9 to 97% interspecies similarity and 0 to 1.7% interspecies divergence to design a primer pair for both PCR amplification and sequencing of this region for identification of rapidly growing mycobacteria [27]. Using these PCRs within the culture negative cohort, both *rpoB* and *ku* PCRs identified the presence of *M. africanum*, *M. bovis,* and *M. orygis* DNA in three separate elephant samples. These samples were also all *ESAT-6/CFP-10*, Ultra, and Hain LPA positive, suggesting that these elephants were truly infected (supplementary material). Agreement analysis for all samples between tests and test combinations versus culture for genus detection and species identification revealed that the *rpoB* PCR is the ideal individual test compared to the rest. The Hain LPA detected slightly more samples containing Mycobacterium genus DNA, but differentiated species very poorly compared to culture. Therefore, combining the *rpoB* PCR with either the *ku* PCR and/or Hain LPA slightly increased genus detection, but not species identification compared to culture. All these findings have significant consequences for species management, public health, veterinary disease control, and conservation endeavours [32,41,42].

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Limitations of this study included the use of Sanger sequencing, as opposed to deep sequencing, resulting in shallow depth coverage, limited sample size, multiple freeze-thawing of samples prior to testing, Hain LPA specialised equipment and training requirements, possible underestimated sensitivities and overestimated specificities when using animal samples. Lastly, the cohort include only animals from *M. bovis* and *M. tuberculosis* endemic areas. Since animals were naturally infected, the sporadic shedding of MTBC and lack of corroborating evidence of NTM infection could have significantly affected the findings in this study. Therefore, additional studies are needed to assess performance of culture-independent techniques for rapid identification of MTBC and NTM infections in livestock and wildlife.

In conclusion, based on this pilot study, the Ultra appeared to be the optimal diagnostic test for MTBC DNA detection directly from raw antemortem respiratory specimens and the *rpoB* PCR for Mycobacterium genus DNA detection and species identification through amplicon sequencing. Notably, the need for an NTM-specific test like the Hain LPA could be circumvented by combined use of the Ultra and *rpoB* PCR.

4. Materials and Methods

4.1. Animals

Throughout 2018, 2019, and 2021, 23 BALF samples were opportunistically collected from free-ranging white (n=21) and black (n=1) rhinoceros from *M. bovis*-endemic KNP and zoo kept black (n=1) rhinoceros. Trunk wash and BALF samples were also opportunistically collected from (n=23) free-ranging *M. bovis* exposed African elephants in KNP and (n=3) zoo kept elephants.

4.2. Antemortem sample collection and mycobacterial culture

Bronchial alveolar lavage fluid was collected endoscopically, and TW samples as previously described [5,43,44]. For optimal *Mycobacteria spp.* isolation, all antemortem respiratory samples were processed for mycobacterial culture using the conventional Mycobacteria Growth Indicator Tubes (MGIT, Becton Dickson, Franklin Lakes, NJ, USA), in parallel with a novel decontamination and mycobacterial culture technique (TiKa) for improved sensitivity, as previously reported [8,31]. Briefly, one aliquot of sample was decontaminated using BBL MycoPrep (Becton Dickinson) and inoculated into conventional MGIT tubes containing BBL MGIT PANTA-OADC enrichment media (Becton Dickinson) as described by the manufacturers. A second aliquot of sample was decontaminated using TiKa-KiC (TiKa Diagnostics, London, UK) and inoculated into MGIT-TiKa tubes containing PANTA-OADC enrichment media and TiKa supplement B reagent (TiKa Diagnostics). All MGITs (conventional and TiKa) were transferred to the BACTEC MGIT 960 mycobacterial detection system (Becton Dickinson). All liquid cultures with detected bacterial growth were sub-cultured onto blood agar plates to exclude contaminants, and subjected to Ziehl-Neelsen (ZN) acid fast staining [45]. All MGITs (conventional and TiKa) with positive bacterial growth underwent further *Mycobacteria spp.* genetic speciation using the region of difference PCR, *16S* rRNA, *rpoB* and *hsp65* PCRs, and Sanger sequencing (Illumina, Inc., San Diago, CA, USA) as previously described [27,28,46]. Animals with samples confirmed to contain either MTBC and/or NTM by culture and genetic speciation were defined as “MTBC positive” or “NTM positive”. Furthermore, the presence or absence of

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immunogenic proteins in all bacterial growth positive MGIT tubes were confirmed by PCR amplification and subsequent amplicon sequencing of *ESAT-6* and *CFP-10* genes as previously described [47].

4.3. Nucleic acid amplification tests (NAAT) for Mycobacteria spp. detection and differentiation

The Xpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, USA) was performed on raw BALF and TW samples for the detection of MTBC DNA as previously described [5]. Briefly, 700 µl of samples were treated to chemical lysis as prescribed by the manufacturer, and 2 ml of the solution aliquoted into Xpert MTBC/RIF Ultra cartridge sample chamber. Result outputs were as follows: 1) "MTB not detected"; 2) "MTB trace detected" and 3) "MTB detected high/medium/low/very low" [5].

Prior to performing the remaining NAATs, total DNA was first extracted from all raw BALF and TW samples using the QIAGEN DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) as previously described [48]. The DNA was used in the Genotype CMdirect version 1.0, line probe assay (Hain Lifescience, Germany) to determine the presence of the *Mycobacterium* genus, MTBC (without differentiation) and subsequent detection and differentiation of more than 20 clinically relevant NTM species, according to manufacturer's instructions. Briefly, multiplex PCRs were performed using biotinylated primers and proprietary amplification mixes in a MiniAmp™ Thermal Cycler (ThermoFisher Scientific, Waltham, MA, USA) with a ramp rate of ≤2.2 ℃/s. Thereafter, amplicons were reverse hybridized in an automated GT-Blot 48 hybridization washer (Bruker, Billerica, MA, USA) onto precoated membrane strips and hybridisation results interpreted according to a template provided within the kit.

Extracted DNA was also subjected to conventional PCR using *Mycobacterium*-specific primers for two highly conserved genetic regions, the *ku* (600 bp) and *rpoB* (740 bp) genes. Amplicons were speciated by Sanger sequencing (Illumina, Inc.) as previously described [26–28]. Briefly, for both targets, a total reaction volume of 25 µl was prepared that consisted of 12,5 µl OneTaq Hot Start 2x master mix (New England Biolabs, Ipswich, MA, USA), 0.8 µM of each respective forward and reverse primer (Integrated DNA Technologies, Coralville, IA, USA), and 6,5 µl nuclease-free water and 5 µL DNA template. Positive controls which included 5 µL 30ng/µl extracted H37Rv *M. tuberculosis* and *M. bovis* DNA, as well as no template controls were included with each PCR. Using a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Waltham, MA, USA), cycling conditions were 95℃ for 15 minutes, followed by 45 cycles at 95℃ for 1 minute, 64℃ for 1 minute and 72℃ for 1 minute, and final elongation at 72℃ for 5 minutes. Amplicon presence, size and intensity were confirmed by 1,5% agarose gel electrophoresis, followed by gel imaging using the ChemiDoc M.D. Universal Hood III Gel Documentation System (Bio-Rad, Hercules, CA, USA). All amplicons were sequenced by Sanger sequencing (Illumina, Inc.) through the Central Analytical Facility (CAF) at Stellenbosch University, SA. Sequence contigs were generated using Sequencher 5.1. software (Gene Codes, Ann Arbor, MI, USA) and blasted on NCBI's Basic Local Alignment Search Tool for nucleotide (BLASTn)[29].

4.4. Data analysis

All positive and negative NAAT and mycobacterial culture results are reported as proportions of the total number of animals and respiratory samples tested. For all known *Mycobacteria* spp. (MTBC and NTM) culture

positive specimens, each relevant NAAT and combination of NAATs with similar target species, the test-positive proportions were compared using a two-tailed z-test where z-scores and *p*-values were calculated (https://www.socscistatistics.com/tests/ztest/default2.aspx). P-values were considered statistically significant if $p<0.05$. Thereafter, agreement analysis was performed between relevant individual NAATs and test combinations for genus detection and differentiation, MTBC and NTM detection and differentiation by calculating Cohen's kappa coefficient (κ) using the online agreement calculator webtool (https://graphpad.com/quickcalcs/kappa1/). All NAATs and culture results for each sample are provided in the supplementary material.

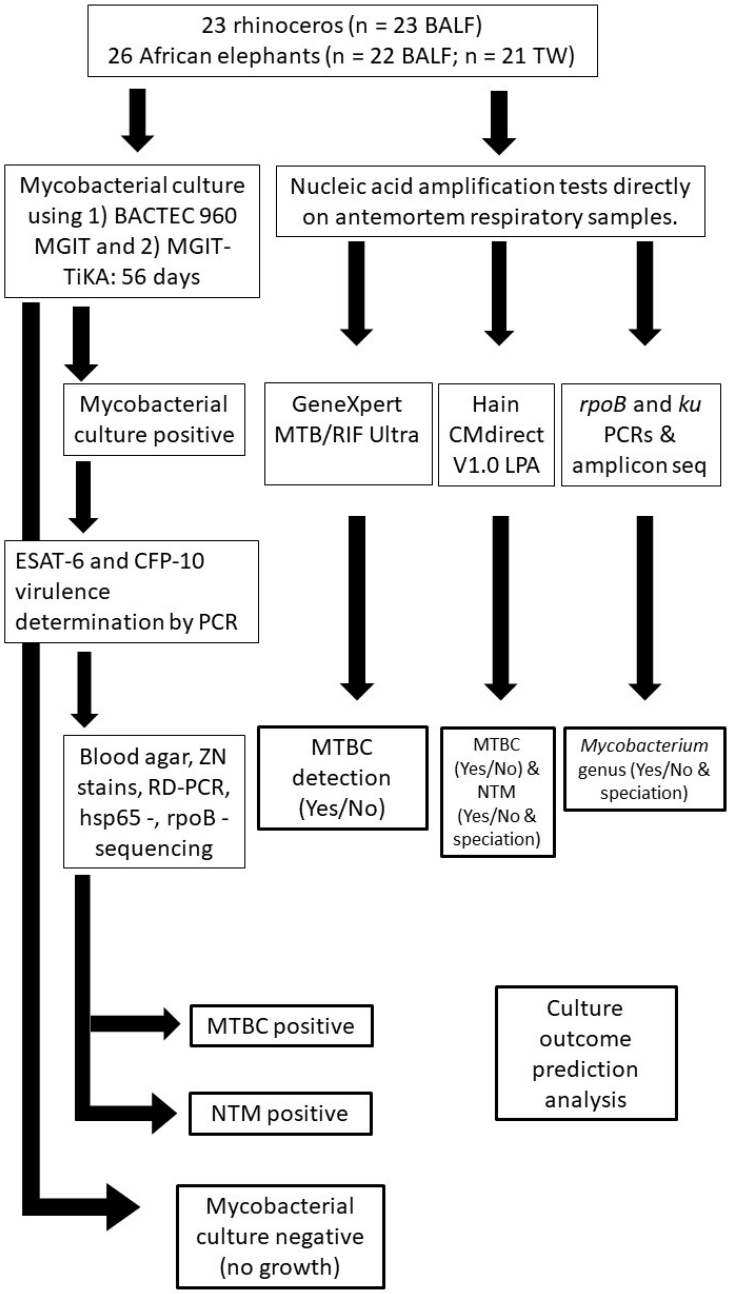

Figure 1. Study method flow chart for African elephants (n=26) and rhinoceros (n=23) respiratory sample processing and PCR testing for mycobacterial identification. TW: Trunk wash; BALF: Bronchioalveolar lavage fluid; RD: Region of difference; MTBC: *Mycobacterium tuberculosis* complex; NTM: Non-tuberculosis mycobacteria.

Supplementary Materials: The following are available online at: [Journal insert location], File S1: All results and metadata.xlsx

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Institutional Review Board Statement: Ethical approval for this study was granted by the Stellenbosch University Animal Care and Use committee (ACU-2021-19019 and ACU-2022-21572). Permission to perform animal research in terms of section 20 of the Animal Diseases Act was granted by the South African Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa (12/11/1/7/2 and 12/11/1/7/6). All animals were handled by the Veterinary Wildlife Services veterinarians and game capture teams of South African National Parks according to their standard operating procedures.

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Data Availability Statement: Data are available in supplementary material

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Conflicts of Interest: The authors declare no conflict of interest.

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Table 1 Mycobacterial isolates identified by culture and virulence determination of n = 20 respiratory samples from 13/26 African elephants and 2/23 rhinoceros, including the combinational use of four nucleic acid amplification tests directly on respiratory specimens for the prediction of culture outcome.

				Mycobacterial culture result from antemortem respiratory specimens		Nucleic acid amplification test results from antemortem respiratory specimens			
Infection status	**Species (#animals)**	**Sample type (Animal ID)**	**Location (South Africa)**	**Combined MGIT and MGIT-TiKa result**	**ESAT-6 & CFP-10 virulence determination**	**Xpert MTB/RIF Ultra result**	**Hain CMdirect V1.0 LPA result**	**rpoB PCR result**	***ku* PCR result**
Confirmed MTBC-infected	African elephants n = 5 Rhinoceros n = 1	BALF (18/85)	Zoo	*M. tuberculosis*	Positive	MTB DETECTED Medium; RIF resistance indeterminate	MTBC	*M. tuberculosis*	*M. tuberculosis*
		BALF (18/177)	Zoo	*M. tuberculosis*	Positive	MTB TRACE DETECTED	mixed NTM	*M. tuberculosis*	*M. tuberculosis*
		BALF (18/177)	Zoo	*M. africanum*	Positive	MTB TRACE DETECTED	*M. avium* and/or *M. interjectum*	*M. africanum*	*M. africanum*
		TW (18/177)	Zoo	*M. elephantis strain*	Positive	MTB NOT DETECTED	*M. fortuitum* group	*M. elephantis*	*M. fortuitum* group
		BALF (18/527)	KNP	*M. stomatepiae*	Negative	MTB NOT DETECTED	MTBC and/or *M. fortuitum* group	*M. stomatepiae*	mixed NTMs - *M. smegmatis*
		TW (18/527)	KNP	*M. bovis*	Positive	MTB TRACE DETECTED	mixed NTM	*M. bovis*	*M. bovis*
		TW (18/533)	KNP	*M. bovis*	Positive	MTB TRACE DETECTED	MTBC and/or mixed NTM infection	*M. bovis*	*M. bovis*
		TW (18/538)	KNP	*M. bovis*	Positive	MTB TRACE DETECTED	*M. fortuitum* group	*M. bovis*	*M. bovis*
		BALF (19/46)	KNP	*M. bovis*	Positive	MTB TRACE DETECTED	MTBC and/or mixed NTM infection	*M. bovis*	*M. bovis*
		TW (18/173)	KNP	*M. avium complex strain*	Positive	MTB TRACE DETECTED	MTBC and/or mixed NTM infection	*M. avium* complex	*Mycobacteria spp.*
		BALF (18/176)	Zoo	*M. foliorum*	Positive	MTB NOT DETECTED	*M. fortuitum* group	*M. fortuitum* group	*M. fortuitum* group
		BALF (19/460)	KNP	*M. mantenii*	Positive	MTB TRACE DETECTED	MTBC and/or mixed NTM infection	*M. mantenii*	*M. mantenii*
		TW (19/460)	KNP	*M. abscessus strain*	Positive	MTB NOT DETECTED	*M. fortuitum* group and/or *M. abscessus*	*M. abscessus*	*M. abscessus*
		BALF (18/530)	KNP	*M. interjectum strain*	Positive	MTB NOT DETECTED	mixed NTM	*M. interjectum strain*	mixed NTMs - *M. avium* complex
		TW (18/530)	KNP	*M. avium strain*	Negative	MTB NOT DETECTED	mixed NTM	*M. avium* complex	*M. elephantis* strain
		TW (18/532)	KNP	*M. mageritense strain*	Negative	MTB NOT DETECTED	*M. fortuitum* group	Negative	Negative
		TW (18/534)	KNP	*M. intracellulare*	Positive	MTB TRACE DETECTED	MTBC and/or mixed NTM infection	*M. avium* complex	*M. intracellulare*
		TW (18/539)	KNP	*M. intracellulare*	Negative	MTB NOT DETECTED	*M. fortuitum* group	*M. intracellulare*	mixed NTMs - *M. avium* complex
		TW (21/496)	KNP	*M. fortuitum strain*	Positive	MTB NOT DETECTED	*M. fortuitum* group	*M. fortuitum*	*M. elephantis* strain
		BALF (18/31)	KNP	*M. scrofulaceum strain*	Positive	MTB NOT DETECTED	mixed NTM	*M. avium subsp. Paratuberculosis*	mixed NTMs - *M. avium subsp. Paratuberculosis*

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BALF: Bronchoalveolar lavage fluid
TW: Trunk wash
KNP: Kruger National Park
MGIT: Measurable growth in the *Mycobacterium* growth indicator tube (MGIT) detected by the BACTEC MGIT 960 mycobacterial detection system and strain typing (Warren et al., 2016).
TiKa: modified MGIT system by alternative use of cationic D-enantiomer peptides for sample decontamination and the additional use of Supplement B during tube inoculation (Goosen et al. 2022).
ESAT-6/CFP-10: PCR amplification of both virulence genetic targets from all cultures.
Xpert MTB /RIF Ultra: MTBC DNA detection through probe-based qPCR that simultaneously targets insertion elements IS6110 and IS1081 and if postive, targets rpoB for drug resistance determination.
Hain CMdirect V1.0 line probe assay is a test system for the detection of M. tuberculosis complex and differentiation of more than 20 clinically relevant NTM directly from patient specimens.
rpoB PCR uses Mycobacterium genus specific primers for the identification of genus DNA and subsequent speciation through amplicon sequencing.
Ku PCR used Mycobacterium genus specific primers for the identification of genus DNA and subsequent speciation through amplicon sequencing.

Table 2 The kappa (κ), 95% confidence interval, standard error (SE) of agreement between four tests and culture for antemortem *Mycobacterium* genus DNA detection from respiratory samples from African elephants (n=26) and rhinoceros (n=23).

Test and combinations	*rpoB* PCR	*ku* PCR	Hain LPA	Culture	rpoB/ku	rpoB/Hain LPA	ku/Hain LPA	rpoB/Ku/Hain LPA
rpoB PCR	1							
ku PCR	0.91 (0.81-1.00, 0.05)	1						
Hain LPA	0.94 (0.85-1.00, 0.04)	0.88 (0.76-0.99, 0.06)	1					
Culture	0.47 (0.29-0.66, 0.09)	0.50 (0.31-0.68, 0.09)	0.32 (0.16-0.48, 0.09)	1				
rpoB/*ku* PCRs	0.97 (0.91-1.00, 0.03)	0.94 (0.86-1.00, 0.04)	0.97 (0.91-1.00, 0.03)	0.45 (0.27-0.63, 0.09)	1			
rpoB/Hain LPA	0.94 (0.85-1.00, 0.04)	0.94 (0.85-1.00, 0.04)	1.00 (0.95-1.00, 0.03)	0.46 (0.29-0.64, 0.09)	0.94 (0.85-1.00, 0.04)	1		
ku/Hain LPA	0.94 (0.85-1.00, 0.04)	0.94 (0.85-1.00, 0.04)	1.00 (0.95-1.00, 0.03)	0.46 (0.29-0.64, 0.09)	0.94 (0.85-1.00, 0.04)	1.00 (0.95-1.00, 0.03)	1	
rpoB/*Ku*/Hain LPA	0.94 (0.85-1.00, 0.04)	0.94 (0.85-1.00, 0.04)	1.00 (0.95-1.00, 0.03)	0.46, (0.29-0.64, 0.09)	0.94 (0.85-1.00, 0.04)	1.00 (0.95-1.00, 0.03)	1.00 (0.95-1.00, 0.03)	1

rpoB PCR uses Mycobacterium genus specific primers for the identification of genus DNA and subsequent speciation through amplicon sequencing.
Ku PCR used Mycobacterium genus specific primers for the identification of genus DNA and subsequent speciation through amplicon sequencing.
Hain CMdirect V1.0 line probe assay is a test system for the detection of M. tuberculosis complex and differentiation of more than 20 clinically relevant NTM directly from patient specimens.
Mycobacterial culture detection based on the combined isolation of Mycobacteria spp. through conventional BACTEC 960 MGIT system and MGIT-TiKA, followed by appropriate genetic speciation of all isolates (Warren et al., 2016).
Landis Kappa scale: Kappa < 0: No agreement; Kappa between 0.00 and 0.20: Slight agreement; Kappa between 0.21 and 0.40: Fair agreement; Kappa between 0.41 and 0.60: Moderate agreement; Kappa between 0.61 and 0.80: Substantial agreement; Kappa between 0.81 and 1.00: Almost perfect agreement."

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