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Genetic Diversity, Population Structure, and Intra-Host Variation of Mycobacterium avium subsp. hominissuis in Fattening Pigs

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Abstract

Background: *Mycobacterium avium*subsp. *hominissuis*(MAH) is an opportunistic environmental pathogen affecting pigs, humans, and occasionally other animal species. Although molecular typing has improved the understanding of MAH epidemiology, information on the genetic diversity and dissemination of MAH in pig populations remains limited. This study investigated the genetic diversity and distribution of MAH in slaughtered fattening pigs in Switzerland using variable-number tandem repeat (VNTR) typing combined with complete *hsp65* gene sequencing.

Results: A total of 490 tissue samples from 120 pigs with granulomatous lesions collected between 2015 and 2022 were examined. Among 342 MAH isolates recovered from 117 pigs, 84 distinct strain genotypes were identified. MAH was detected in tissues with and without granulomatous lesions, including five macroscopically unremarkable muscle samples obtained from pigs in which MAH was also detected in other organs. Eight strain genotypes accounted for 203 of 342 isolates (59.4%), whereas 52 of 84 strain genotypes were represented by a single isolate. Mixed infections were common, with 56 of 117 pigs (47.9%) harbouring two or more strain genotypes and up to six detected within a single animal. Dominant strain genotypes occurred across multiple farms, cantons, and sampling years, while epidemiological clusters showed no significant geographic clustering. Comparison with previously published data identified closely related strain genotypes among isolates from pigs, cattle, and humans from several countries.

Conclusions: The MAH population was characterized by a high genetic diversity, frequent mixed infections, and the coexistence of a small number of geographically widespread strain genotypes alongside numerous rare variants. The observed population structure is consistent with repeated environmental exposure as the principal driver of MAH epidemiology in pigs. The occurrence of closely related strain genotypes across animal species and humans further supports the relevance of MAH from a One Health perspective.

BACKGROUND

Mycobacterium avium subsp. *hominissuis* (MAH) is an opportunistic pathogenic bacterium belonging to the *Mycobacterium avium* complex (MAC) within the genus *Mycobacterium* [1]. It primarily infects pigs and humans, but can also cause opportunistic infections in other mammals, including cattle [1, 2]. In pigs, MAH infections are typically subclinical and therefore often remain undetected until slaughter. In contrast, humans, particularly immunocompromised individuals, are susceptible to clinical infection, with MAH frequently causing pulmonary disease, soft tissue infection, lymphadenitis or disseminated infections [3, 4].

Nontuberculous mycobacteria (NTM) such as MAH are known to be ubiquitously present in the environment, including soil, water and dust [5, 6]. Transmission is thought to occur mainly via environmental exposure rather than direct contact between infected individuals; however, zoonotic transmission through contaminated meat or direct contact cannot be excluded [7–9]. In pigs, infection may lead to granulomatous lesions, particularly in lymph nodes associated with the gastrointestinal tract. MAH can also be present in lymphatic tissues without causing visible lesions, and dissemination to organs such as the lungs and liver has been reported [8]. Surveillance in pigs relies primarily on routine meat inspection based on the visual detection of granulomatous lesions. Although generally effective, the subjective nature of visual inspection may result in failure to detect early lesions [10]. When granulomatous lesions are detected during inspection, carcasses are typically condemned in accordance with meat inspection regulations due to suspicion of mycobacterial infection, resulting in economic losses for farmers of approximately CHF 200 – 250 per animal [11, 12]. Additionally, MAH infection may lead to wasting, reduced fertility and abortion in sows, occasionally presenting with clinical signs such as abortion [13, 14]. Therefore, understanding the epidemiology of MAH is of both public health and economic importance, particularly in countries such as Switzerland, where pig production relies on a highly interconnected production system.

In Switzerland, this economic relevance is particularly important because pork represents the most widely consumed meat, with an average annual consumption of approximately 20 kg per capita[15]. To meet this demand, approximately 2.5 million pigs are slaughtered annually [15]. Around 92% of the pork available on the Swiss market originates from domestic production, reflecting the economic importance of the national pork industry [15]. Swiss pig production is organized as a hierarchical production pyramid in which a relatively small number of breeding herds supply numerous birth farms, which subsequently distribute piglets to rearing and fattening farms [16]. This highly interconnected production system facilitates efficient pork production but also enables pathogens introduced at an early production stage to be disseminated throughout the production chain via animal movements. Therefore, tracing infection pathways can be challenging, as pigs may be exposed to infectious agents through both direct transmission associated with animal movements and repeated environmental exposure at multiple stages before slaughter. Recent network analyses further demonstrated that the Swiss pig production system has become increasingly connected over time, potentially increasing opportunities for infectious disease exposure and emphasizing the importance of understanding transmission pathways within the production chain [17]. Consequently, accurate detection, identification and molecular characterization of MAH are essential for understanding transmission pathways and developing control strategies.

Historically, identification of members of the MAC relied on culture characteristics and biochemical tests, which lacked sufficient discriminatory power to reliably distinguish closely related species and subspecies within the complex [18]. Molecular methods therefore became essential for accurate species identification and epidemiological investigations. At the strain level, methods such as IS1245 restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) provided improved discriminatory power. However, these methods were labour-intensive and difficult to standardize between laboratories, leading to the development of PCR-based and sequencing-based approaches [19, 20]. Consequently, PCR- and sequencing-based approaches, including heat shock protein (*hsp65)* gene sequencing and variable number tandem repeat (VNTR) analysis, have become the most widely used tools for MAH strain characterization and epidemiological investigations in veterinary medicine, although whole-genome sequencing (WGS) is increasingly being adopted for high-resolution analyses.

VNTR typing enables discrimination between bacterial strains based on variation in repeat numbers at specific genomic loci. Originally developed as Mycobacterial interspersed repeat unit (MIRU)-VNTR analysis for *Mycobacterium tuberculosis*, the method was subsequently adapted for *M. avium* through

the identification of *Mycobacterium avium* tandem repeat (MATR) loci, resulting in the combined MIRU/MATR typing schemes widely used for MAH epidemiology [19, 21, 22]. The combination of repeat numbers across all analysed loci generates a strain-specific **allelic profile**, which can be used to compare isolates and assess epidemiological relationships [19, 23]. A standardized set of VNTR markers for MAH was established by Thibault et al. 2007, providing a robust framework for molecular epidemiological studies [19]. Since then, VNTR typing has been widely applied to characterize MAH isolates from pigs, cattle, humans, and even environmental sources, enabling studies of strain diversity, spreading mechanisms and population studies [8, 19, 23–26]. Compared with earlier methods, VNTR analysis is simple, reproducible, and comparable between laboratories, making it suitable for large-scale epidemiological studies [23, 24].

This study aimed to (i) identify the mycobacterial species associated with granulomatous lymph node lesions in Swiss fattening pigs, (ii) to characterize the genetic diversity and spatial distribution of MAH strains throughout the Swiss pig production chain using an optimized VNTR typing scheme combined with complete *hsp65* gene sequencing, and (iii) investigate strain diversity at the farm, animal, and organ levels to improve understanding of MAH transmission and dissemination. Finally, the identified strain genotypes were compared with previously published animal and human isolates to evaluate the zoonotic potential and global occurrence.

METHODS

Sample Collection

A total of 490 tissue samples from 120 fattening pigs slaughtered at an abattoir in western Switzerland were collected between 2015 and 2022, representing 13 of the 26 Swiss cantons. Animal carcasses were selected for sampling when they exhibited granulomatous lesions in one or more lymph nodes at post-mortem inspection. Most samples (469 tissues from 100 pigs; 95.7%) were collected between 2020 and 2022. The remaining 21 tissue samples originated from 20 pigs sampled in 2015 as part of the Swiss national Lymph Node Monitoring (LyMON) project and were included retrospectively [32].

Whenever possible, five anatomical sites were sampled from each animal, including mandibular and mesenteric lymph nodes, liver, lung, and muscle. Samples included both tissues with granulomatous lesions and macroscopically unremarkable tissues. The resulting sample set comprised 94 muscle, 93 liver, 92 lung, 89 mandibular lymph node, and 88 mesenteric lymph node samples. In addition, eleven pooled lymph node samples (mandibular, mesenteric and/or retropharyngeal) and two pooled liver and spleen samples were collected. Owing to the retrospective nature of the 2015 collection, tissue sampling was less comprehensive than during the later sampling period, with fewer tissues collected per animal (mean = 2.75/pig) and all available samples submitted as pooled tissues rather than as individual anatomical specimens. Samples were transported to the laboratory in chilled 250 ml multi-purpose containers (Sarstedt, Nümbrecht, Germany).

Sample Preparation

Animal samples were processed as described previously [27]; Briefly, 2 g of each tissue sample were homogenized in 20 ml of 0.9% saline solution using a rotating-blade macerator (T18 Digital Ultra-Turrax, IKA, Staufen, Germany), followed by centrifugation at 3000 × g for 15 min. After removal of the supernatant, the pellet was decontaminated by resuspension in 4 ml of 4% H_2SO_4, incubated for 15 min at room temperature, and subsequently neutralized with 5.65 ml of 1 M NaOH. Next, 20 ml of phosphate-buffered saline (PBS, pH 7.4) were added, and the suspension was centrifuged again at 3000 × g for 15 min. The resulting pellet was resuspended in 3 ml PBS and used as inoculum.

Mycobacterial Culture

Mycobacterial culture was performed as described previously [27]. Two BBL MGIT liquid culture tubes, supplemented with BACTEC MGIT 960 growth supplement, the BBL MGIT PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; BD, Allschwil, Switzerland), and 50 µg/ml sodium pyruvate, were each inoculated with 0.5 ml of the prepared inoculum. The tubes were incubated at 37°C for up to 8 weeks in a BACTEC MGIT 320 system (BD) and monitored regularly for microbial growth.

To obtain pure mycobacterial isolates, subcultures were performed on 7H10 agar and BBL Stonebrink agar slants (BD). Cultures exhibiting growth consistent with mycobacteria were further examined for acid-fast bacilli using Ziehl–Neelsen staining. A total of 421 mycobacterial isolates were recovered and included in the analyses.

DNA Extraction

DNA from cultured mycobacteria was extracted by inoculating a loop-full of cell materials into 200 µl of chelating ion-exchange resin (InstaGene Matrix) and centrifuged at 13,000 g for 10 min. The supernatant was used in downstream reactions. DNA concentration in the final eluate was determined by measuring absorbance at 260 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Reinach, Switzerland). Samples were then diluted to a final concentration of 10 ng/µl and stored at −20°C until further use.

Species Identification and genotyping

Species identification was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for species identification, as described previously [28]. Briefly, bacterial material from cultures grown on 7H10 agar was inactivated by heat treatment and subjected to ethanol–formic acid extraction ((MycoEX, Bruker Daltonics, Bremen, Germany). Extracts were spotted onto a MALDI target plate, overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) matrix, and analyzed using a Microflex LT system (Bruker Daltonics).

Spectra were acquired in linear positive ion mode and analyzed using the MALDI Biotyper Compass software (Compass IVD, version 4.2.90.7; Bruker Daltonics) with reference to the Mycobacteria Library (Myco 7, 1069 reference spectra). Identification was based on log score values according to the manufacturer's criteria.

Heat shock protein *(hsp65)* Sequencing

After initial identification as *Mycobacterium avium* by MALDI-TOF MS, the complete *hsp65* gene was amplified and sequenced for all confirmed isolates to enable subspecies-level differentiation, following the protocol described previously [29]. Sanger sequencing was performed at Microsynth (Balgach, Switzerland). Sequence assembly and analysis were conducted using ApE [30] and FinchTV [31].

MATR/MIRU-VNTR Genotyping and Terminology

MATR- resp. MIRU-VNTR typing was performed as previously described [19, 22]. The following loci were analysed: MATR-1, MATR-2 (MIRU-292), MATR-3 (MIRU-X3), MATR-4 to MATR-9, MATR-11 to MATR-16, as well as MIRU-3, MIRU-7, MIRU-25, MIRU-37, and MIRU-47. Primers targeting the flanking regions of each VNTR locus are listed in **Additional File 1: Table S1**.

PCR amplification was carried out in a total volume containing 5 µl TaqPCR Master Mix (Qiagen), 2 µl Q-Solution (Qiagen), 0.5 µl primer mix (forward and reverse), 0.5 µl nuclease-free water, and template DNA at a concentration of 10 ng/µl. Cycling conditions consisted of an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 30 s at 95°C, 60°C, and 72°C, and a final extension step at 72°C for 10 min.

10 µl of each PCR amplification product were analyzed using a capillary electrophoresis device (QIAxcel, Qiagen), using the OH1700 AM10sec method with a QX DNA high-resolution cartridge, QX 15 bp– 3 kb alignment marker and QX 100bp– 2.5 kb size marker. Peak size assignment and allele code exportation was performed with the QIAxcel ScreenGel Software version 1.3.0 (Qiagen).

VNTR profiles were used to define strain genotypes and assess genetic relatedness among isolates. Throughout this study, an **isolate** refers to a cultured *Mycobacterium avium* subsp. *hominissuis* (MAH) colony recovered from a single tissue sample. Multiple isolates could therefore originate from the same animal or, in some cases, from the same tissue. The term **strain genotype** refers to MAH isolates sharing identical allelic profiles across all included VNTR loci and the *hsp65* sequence variant. Isolates differing at one or more loci were assigned to different strain genotypes. For epidemiological analyses, closely related strain genotypes were grouped into epidemiologically linked clusters using a threshold of ≤1 allelic difference among isolates collected during 2020–2022 and ≤2 allelic differences for comparisons between isolates collected in 2015 and 2020–2022. Genetic distances were calculated using a binary allelic difference matrix in which each locus contributed a distance of 0 (identical allele) or 1 (different allele). Missing loci were retained and treated as a fixed allelic difference of 1.

Data Analysis

A total of 421 mycobacterial isolates were obtained, of which 79 were excluded because of contamination, mixed cultures, or incomplete VNTR typing results. The final dataset comprised 342 MAH isolates. One isolate showing evidence of a mixed culture was retained in the final dataset but excluded from epidemiological cluster assignment because it could not be unambiguously assigned.

All statistical analyses and data visualizations were performed in R (version 4.5.1). Diversity analyses and multivariate statistics were conducted using the vegan package, minimum spanning trees were generated using igraph, and figures were prepared using ggplot2.

Minimum spanning trees (MSTs) were constructed from the binary allelic distance matrix to visualize genetic relationships among strain genotypes and epidemiological clusters and to evaluate the contribution of complete *hsp65* sequence information to strain discrimination. For each strain genotype, the number of neighbouring strain genotypes differing by ≤2 allelic-profile differences was determined. Associations with strain abundance were assessed using Pearson's and Spearman's correlation coefficients.

The spatial distribution of sampled pigs was visualized using geographic coordinates obtained from the Swiss Animal Movement Database (Tierverkehrsdatenbank, TVD), including birth farms and the last recorded fattening farms before slaughter. The geographic distribution of the dominant strain genotypes was investigated with respect to the number of affected pigs, farms, cantons, and years of detection. Geographic clustering of epidemiological clusters was assessed separately for birth and fattening farm locations using a permutation-based spatial compactness analysis. Only clusters represented by at least three animals were included, and observed median pairwise geographic distances were compared with those obtained from 5,000 random permutations of the same number of sampled locations. The association between cluster size and geographic distribution was assessed using Spearman's rank correlation. In addition, pairwise farm comparisons were performed to evaluate whether farms located within predefined distance thresholds (10, 20, and 50 km) were more likely to share identical strain genotypes or epidemiological clusters than more distant farms. Associations were quantified using odds ratios with 95% confidence intervals, and *P*-values were adjusted for multiple testing. Farm-level strain diversity was quantified using the Shannon diversity index, and the association between sampling intensity and strain diversity was assessed using Spearman's rank correlation. Differences in allelic profiles among birth farms, fattening farms, and individual animals were assessed using permutational multivariate analysis of variance (PERMANOVA; *adonis2*, **vegan** package) with 999 permutations. Within-host genetic diversity was further characterized using pairwise allelic distances among isolates recovered from the same animal. Associations between strain genotype and dissemination pattern (generalized versus localized infection) were assessed using contingency table analysis. Fisher's exact test was used when expected cell frequencies were too low for a chi-square test; otherwise, the chi-square test was applied.

RESULTS

Aetiology of Granulomatous Lymph Node Lesions in Fattening Pigs

The study population comprised 120 pigs originating from 64 recorded birth farms and submitted for slaughter from 75 different fattening farms. Birth farm information was unavailable for seven animals. Most birth farms were represented by a single pig (median = 1 pig/farm, mean = 1.81 pigs/farm), with the largest birth farm contributing eight pigs. A total of 35 birth farms (54.7%) were represented by only one sampled animal. Similarly, fattening farms were represented by a median of one pig (mean = 1.56 pigs/farm), with a maximum of six pigs originating from a single fattening farm. Fifty-one fattening farms (68.0%) were represented by only one sampled animal.

Over the study period, 13–44 animals were sampled annually (**Figure 1A**), resulting in the collection of 20–205 tissue samples and 20–140 cultured isolates per year. (**Figure 1B and C**). The number of distinct strain genotypes identified per year ranged from 14 to 43 (**Figure 1D**).

Culture and MALDI-TOF MS results were available for the 469 tissue samples from the second sampling periods. MAH was identified in 308 tissue samples (65.7%) and was detected in at least one tissue from 97 of the 100 pigs (97.0%). Three pigs yielded no MAH isolates and were therefore excluded from the molecular typing analyses. Together with the MAH isolates obtained in 2015 and additional subcultures derived from mixed cultures, this resulted in a final molecular typing dataset comprising 342 MAH isolates from 117 pigs. Among the different tissues tested, mesenteric lymph nodes showed the highest proportion of MAH-positive samples (83/88; 94.3%), followed by mandibular lymph nodes (77/89; 86.5%), liver (73/93; 78.5%), lung (58/92; 63.0%). Although MAH was detected predominantly in lymph nodes and parenchymal organs, it was also isolated from five muscle samples without macroscopically visible lesions (5.3%, 5/94). In all five cases, MAH was additionally isolated from at least two other anatomical sites, including lymph nodes and/or parenchymal organs.

Non-tuberculous mycobacteria (NTM) other than MAH were detected in 60 of 469 tissue samples (12.8%; **Additional File 1: Table S2**). Eighteen samples (3.8%) yielded MAH together with one or more additional NTM species, whereas 42 samples (9.0%) yielded only non-MAH NTM. The most frequently detected species were *Mycobacterium fortuitum* (n=30; 6.4% of samples), followed by *M. peregrinum* (19; 4.1%) and *M. neoaurum* (10; 2.1%). *Mycobacterium septicum* was detected twice (0.4%), whereas *M. nebraskense*, *M. lentiflavum*, *M. porcinum* and *M. bohemicum* were each detected once (0.2%). One muscle sample yielded *Corynebacterium falsenii*, and eight cultures were altogether negative for mycobacteria.

Genetic diversity of MAH in Switzerland

VNTR-Typing and *hsp65* Sequencing.

Evaluation of the typing scheme identified four non-informative VNTR loci (MATR-13, MIRU-3, MIRU-7, and MIRU-37), which were excluded from subsequent analyses. Details are provided in **Additional File 1: Text T1** and **Table S3**. The final allelic profile consisted of 15 VNTR loci combined with complete *hsp65* gene sequencing and achieved a high discriminatory power (Hunter-Gaston Discriminatory Index (HGDI) = 0.960), compared with the established INMV typing scheme (HGDI = 0.71). All subsequent analyses were based on this optimized typing scheme.

Strain Definition, Diversity and Clustering

Application of the optimized typing scheme identified 84 distinct strain genotypes among the 342 MAH isolates (**Additional File 1: Table S 4**). Complete *hsp65* sequence data enhanced strain discrimination by distinguishing isolates with identical VNTR profiles into distinct strain genotypes when their *hsp65* sequence codes differed (**Additional File 2: Figure S1)**.

Eight dominant strain genotypes (STR45, STR85, STR133, STR171, STR197, STR223, STR258, and STR301), each represented by more than 10 isolates, accounted for 203 of the 342 isolates (59.4%) and were detected in 83 pigs originating from 59 fattening farms. In contrast, 52 of the 84 strain genotypes (61.9%) were represented by a single isolate, accounting for 15.2% of all isolates. The genetic relationships among strain genotypes are shown in the MST generated from the optimized allelic profiles (**Figure 3)**. The MST showed that many less common strain genotypes were connected to dominant strain genotypes, forming clusters of closely related variants. This pattern was supported by a positive correlation between strain abundance and the number of neighbouring strain genotypes differing by ≤2 alleles (Spearman's $\rho = 0.331$, $P = 0.002$; Pearson's $r = 0.434$, $P < 0.001$).

Using predefined clustering criteria requiring identical *hsp65* sequence codes and a maximum of one allelic difference between isolates collected during 2020–2022 or two allelic differences for comparisons between isolates collected in 2015 and 2020–2022, 58 epidemiological clusters were identified (**Additional File 1: Table S5**). Twenty-eight clusters (48.3%) consisted of a single isolate, while most of the remaining clusters contained two or three isolates. The three largest clusters, C47, C51 and C1, comprised 78 (22.8%), 60 (17.5%) and 51 (14.9%) of the 342 isolates, respectively, together accounting for 189 isolates (55.3%). The largest epidemiological clusters were centred on the dominant strain genotypes STR301, STR223 and STR45. Cluster C47 was detected in 32 pigs from 19 birth farms and 23 fattening farms, cluster C51 in 27 pigs from 21 birth farms and 23 fattening farms, and cluster C1 in 28 pigs from 20 birth farms and 21 fattening farms. The minimum spanning tree based on the epidemiological cluster profiles demonstrated a comparable overall population structure, with the same dominant strain genotypes forming the largest genetic groups (**Additional File 2: Figure S2**).

Spatial Distribution of Strain Genotypes

Birth and fattening farms were distributed across large parts of Switzerland, covering 13 and 11 cantons, respectively (**Additional File 3: Figure S3**). The geographical distribution of sampled farms was influenced by the location of the slaughterhouse; consequently, not all Swiss cantons were represented equally. Farms were geographically dispersed, and mapped animal movements showed links both within and between cantons. The median Euclidian distance between birth and fattening farms was 13.7 km (0.0 – 136.5 km) and 36.8 km (11.5 – 128.0 km) between fattening farm and slaughterhouse.

The spatial distribution of sampled pigs varied between sampling years (**Figure 4**). Sampled animals originated from multiple regions of Switzerland, with repeated movements between the same birth and fattening farm pairs observed in 2015, 2020, and 2022. Among the 84 identified strain genotypes, only four (STR45, STR223, STR258, and STR301) were detected in all four sampling years. These strain genotypes were also recovered from multiple recorded birth and fattening locations across several cantons (**Additional File 1: Table S6**).

Birth locations represented by pigs distributed to two or more different fattening farms were identified and examined for shared MAH strain genotypes (Table 1). Eleven recorded birth locations fulfilled these criteria, comprising 43 pigs. Among these, four birth locations yielded five observations in which pigs (n=9) assigned to different fattening farms shared an identical MAH strain genotype. In each case, the pigs shared only a single MAH strain genotype; no pair of pigs shared multiple strain genotypes. Additional strain genotypes recovered from the affected pigs were compared with the shared strain genotype. In four of 12 unique strain-pair comparisons, the additional strain differed by only 1–2 allelic-profile differences from the shared strain (STR258–STR301, n = 2; STR45–STR37, n = 1; STR183–STR301, n = 1).

To investigate whether MAH exhibited geographic clustering, complementary spatial analyses were performed. Individual epidemiological clusters were not geographically closer than expected by chance, indicating no evidence for localized spatial clustering. Consistent with this observation, larger epidemiological clusters were detected across increasingly broad geographic areas (Spearman's ρ = 0.64, $P < 0.001$), with the four largest clusters spanning 20–39 recorded farm locations and median pairwise distances of 37–53 km. Initially, pairwise comparisons showed that fattening farms located within 20 km of one another were more likely to share identical strain genotypes (31.3% versus 22.5%; OR = 1.56, 95% CI 1.22–2.00) and epidemiological clusters (39.6% versus 31.5%; OR = 1.43, 95% CI 1.13–1.80) than farms separated by greater distances. However, permutation analyses accounting for the spatial dependence of farm pairs did not support a significant association between geographic proximity and genetic similarity.

Table 1. **Identical *M. avium* subsp. *hominissuis* strain genotypes among pigs originating from the same birth location.**

Birth farm	Birth canton	Shared Strain Genotype	Animal ID	Fattening Farm	Fattening canton	Additional Strains in Fattening Farm	Closes
Bfarm009	BE	STR258	118	LFarm044	BE	STR3, STR12	-
			99	LFarm021	FR	STR149, STR301	STR 3
Bfarm11	BE	STR45	7	LFarm043	BE	-	-
			19	LFarm039	BE	STR37, STR112, STR154	STR37
Bfarm12	BE	STR183	63	LFarm007	BE	-	-
			78	LFarm073	BE	STR43, STR108, STR301, STR16	STR30
		STR113	25	LFarm066	BE	-	-
			78	LFarm073	BE	s.o.	-
Bfarm15	FR	STR301	39	LFarm010	JU	STR15, STR154	-
			46	LFarm074	FR	STR258	STR25

Pigs originating from the same recorded birth location but assigned to different fattening farms are shown. For each observation, the shared strain genotype, animal identifier, fattening farm, additional strain genotypes recovered from the same animal, and the closest related additional strain genotype are provided. Relatedness was determined using the optimized allelic profile, including the complete *hsp65* sequence; values in parentheses indicate the number of allelic-profile differences.

Temporal Distribution of Strain Genotypes

Annual sampling intensity and strain diversity varied across the study period (**Figure 1**). The number of sampled pigs ranged from 13 in 2021 to 44 in 2020, while the number of MAH isolates ranged from 20 in 2015 to 140 in 2020 (**Figure 1**). Between 14 and 43 distinct strain genotypes were detected per year. Most strain genotypes were detected in only one sampling year (71/84), whereas four strain genotypes, ST45, ST223, ST258 and ST301, were detected in all four sampled years.

Retrospective inclusion of the 20 isolates collected in 2015 identified nine additional strain genotypes, whereas the remaining 11 isolates belonged to strain genotypes that were also detected between 2020 and 2022. Longitudinal analysis of the four farms sampled at both time points revealed no persistence of identical strain genotypes between sampling periods. Instead, strain pairs differed by more than two alleles. This pattern was not explained by animal origin, as pigs from the same birth location also carried different strain genotypes.

Additional analysis of recurring strains within farms revealed 12 occasions on which the same strain genotype was detected in more than one animal, affecting nine of 75 fattening farms. Repeated detection of the same strain genotype across different sampling years was observed for STR258 and STR301. In only one instance, STR258 was recovered in two different years from pigs assigned to both the same birth and the same fattening farm.

Farm-Level Diversity

Substantial genetic diversity was observed among pigs originating from individual birth and fattening farms. Multiple strain genotypes were identified among pigs assigned to 45 of 75 fattening farms (60.0%) and 44 of 64 birth farms (68.8%), with up to 12 distinct strain genotypes associated with a single far*m.* The majority of the 84 detected strains were rare and detected only in pigs originating from a single fattening farm (63/84). Only a limited number of strains were

widely distributed, with strain genotypes ST301 and ST45 detected in up to 20 farms each and strain genotypes ST223, ST258, and ST133 identified in 10, 11, and 12 farms, respectively (**Additional File 1: Table S6**).

Representative strain and cluster distributions for the ten most extensively sampled birth and fattening farms are shown in **Figure 5** and **Additional File 4**. The number of distinct strain genotypes detected per farm ranged from one to twelve among birth farms (mean = 2.85) and fattening farms (mean =2.42). In six of the ten birth farms and four of the ten fattening farms, more strain genotypes than sampled pigs were detected, indicating that multiple strain genotypes were recovered from individual animals.

Across the complete dataset, pigs originating from 41 of 75 fattening farms included at least one animal infected with multiple strain genotypes, whereas only nine farms were represented by more than one pig carrying the same strain genotype. Similar patterns were observed when strain genotypes were grouped into epidemiological clusters (**Additional File 4: Figure S4**).

Consistent with these observations, Shannon diversity indices varied considerably among farms. While some farms were associated predominantly with a single strain genotype, others were associated with substantially greater strain diversity. A strong positive correlation was observed between Shannon diversity and the number of sampled animals per farm (Spearman's $\rho = 0.79$, $p < 0.001$), indicating that farms with more sampled animals tended to exhibit greater observed strain diversity.

Animal-Level Diversity

Considerable intra-host diversity was observed among sampled pigs (**Table 1**; **Additional File 1 Table S7 and S8**). Of the 117 animals included in the analysis, 30 (25.6%) harboured a single strain genotype confined to a single tissue, whereas 31 (26.5%) carried a single strain genotype detected in multiple tissues. The remaining 56 animals (47.9%) harboured two or more distinct strain genotypes, with up to six strain genotypes identified within a single animal. Isolates recovered from the same pig exhibited significantly more similar VNTR profiles than isolates from different pigs (PERMANOVA: $R^2 = 0.706$, $p = 0.001$). Among the 56 pigs harbouring multiple strain genotypes, 10 (17.9%) contained at least one pair of strain genotypes differing by a single allele and 16 (28.6%) contained at least one pair differing by two alleles. In contrast, 27 pigs (48.2%) harboured at least one pair differing by five or more alleles.

Representative intra-host infection patterns are illustrated in **Figure 6**, including localized infection, detection of the same strain across multiple tissues, and mixed infections involving multiple strain genotypes either in different anatomical sites or within the same tissue.

Organ Distribution and Dissemination Potential

More than one strain genotype was detected in at least one lymph node or parenchymal organ in 22 of the 117 pigs (18.8%). In six pigs, multiple organs harboured two different strain genotypes. The greatest within-animal diversity comprised six distinct genotypes recovered from a single animal. In all remaining cases involving multiple organs, the same two strain genotypes were recovered repeatedly. Pairwise comparison of strain genotypes recovered from the same organ revealed allelic-profile differences ranging from 1 to 11 loci (median = 3.5). Only four of the 30 comparisons (13.3%) differed by a single allelic-profile difference, whereas most co-occurring strain pairs differed by three or more allelic-profile differences (25/30; 83.3%).

Samples from each animal were evaluated for evidence of generalized infection, defined as recovery of the same strain genotype from more than one tissue within an individual animal. Generalized infection occurred across multiple strain genotypes (29/84; 34.5%). Among the more frequently detected genotypes, the proportion of generalized infections ranged from **41.7% (5/12) for STR301** to **68.8% (11/16) for STR223**, while STR85 (4/5), STR171 (3/3), and STR183 (3/3) were also repeatedly associated with generalized infection. However, the frequency of generalized infection did not differ significantly among genotypes (global Fisher's exact test, $P = 0.979$).

One Health Implications

Comparison with INMV Database

Application of the INMV typing scheme resulted in a substantial reduction of the observed strain diversity, with the 84 strain genotypes identified in the present study collapsing into 34 INMV profiles (**Additional File 1: Table S9**). Sixty profiles comprising 302 isolates could be assigned to previously described INMV types, whereas 18 profiles did not correspond to any entry in the reference database.

The most frequently detected profiles corresponded to INMV92 (88 isolates), INMV51 (76 isolates), INMV107 (76 isolates), and INMV98 (17 isolates). Overall, the optimized typing scheme used in the present study provided substantially higher discriminatory power (HGDI = 0.960) than the INMV scheme (HGDI = 0.710), allowing improved differentiation of closely related MAH strains.

Comparison with strains previously described by Scherrer et al (2018) identified two strain genotypes from the present study, STR30 (n = 9 isolates) and STR45 (n = 37), that were identical across all 15 loci to the bovine strains NTM34 and NTM46, respectively [26]. Both strains belonged to the INMV92 lineage and were also identical to human isolates previously reported by Ichikawa et al. (2015), see **Table 3**.

Abbreviations

Abbreviation
Definition

HGDI
Hunter-Gaston discriminatory index
hsp65
Heat shock protein 65 gene
INMV
INRA Nouzilly MIRU-VNTR Profile
MAC
Mycobacterium avium complex
MAH
Mycobacterium avium subsp. *hominissuis*
MALDI-TOF MS
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MATR
Mycobacterium avium Tandem Repeat
MIRU
Mycobacterial interspersed repetitive uni
MST
Minimum spanning tree
NTM
Non-tuberculous mycobacteria
PCR
Polymerase Chain Reaction
PFGE
Pulsed-field gel electrophoresis
TVD
Tierverkehrsdatenbank
VNTR
Variable-numer tandem repeat
WGS
Whole-genome sequencing

DISCUSSION

This study identified MAH as the predominant mycobacterial species associated with granulomatous lymph node lesions in pigs and demonstrated substantial genetic diversity despite the widespread distribution of a limited number of dominant strain genotypes. Furthermore, the observed strain diversity at the farm, animal, and organ levels provides new insights into the transmission and dissemination of MAH within the Swiss pig production system. MAH was detected in at least one tissue sample from 117 of the 120 pigs (97.5%) with granulomatous lesions included in this study. The high detection rate supports the established role of MAH as the predominant NTM associated with granulomatous lymphadenitis in pigs [33]. Nevertheless, a range of other NTM species, including *Mycobacterium neoaurum*, *M. fortuitum*, *M. peregrinum*, *M. septicum*, *M. bohemicum*, and *M. porcinum*, were detected in a subset of samples, either together with MAH or in the absence of MAH. Most of these species belong to the rapidly growing mycobacteria (RGM) which are widely distributed in environmental reservoirs such as soil and water and are generally considered opportunistic pathogens [34, 35]. Given their low frequency of detection and the presence of MAH in all affected animals, these organisms were unlikely to represent the primary cause of the observed lesions and may instead reflect transient colonialisation, environmental contamination or co-infection [33]. In addition, *Corynebacterium falsenii* was isolated from a single muscle sample. Its relevance to the observed lesions in pigs remains uncertain. However, *Corynebacterium falsenii* has been reported to cause clinically significant bacteraemia in a human infant, suggesting that the species may represent an opportunistic pathogen in humans [36]. The clinical relevance of the detected NTM in pigs is currently unknown. Nevertheless, the presence of potentially opportunistic mycobacterial species in slaughter pigs raises questions regarding the possible public health relevance of pork as source of human exposure.

Interestingly, MAH was recovered not only from lymph nodes and parenchymal organs but also from a small number of muscle samples. Similar findings have been reported both in experimentally infected pigs as well as in pork products using real-time PCR, however, detection in muscle appears to be uncommon [7, 37]. This study successfully recovered viable MAH from the muscle tissue of naturally infected pigs by culture, demonstrating that dissemination to edible tissues can occur during natural infection. Although the number of samples was rather low and no conclusions regarding consumer risk can be drawn, this finding may be relevant from both meat inspection and One Health perspectives and warrants further investigation.

The MAH population analysed in this study demonstrated substantial genetic diversity, with 84 distinct strain genotypes identified among 342 isolates. Eight dominant strain genotypes were represented by more than ten isolates each and together accounted for the majority of isolates, whereas many strain genotypes were detected only once (n = 52; 62 %). The repeated detection of dominant strain genotypes such as STR45 and STR301 across multiple farms indicates that some MAH strains are more widely distributed within the Swiss pig production system than others.

Similar population studies characterized by a limited number of common strain genotypes have previously been reported in MAH populations from pigs and other hosts [25, 26, 38]. The MST generated from the optimized allelic profiles (**Figure 2**) further illustrated this pattern, with numerous rare strain genotypes

separated by only one or two allelic differences from the dominant strain genotypes.

Interestingly, the largest epidemiological clusters were centred on the dominant strain genotypes STR301, STR223 and STR45. Consistent with this observation, strain abundance was positively correlated with the number of closely related strain genotypes (Spearman's ρ = 0.331, $P = 0.002$; Pearson's $r = 0.434$, $P < 0.001$). Together, these findings suggest that dominant strain genotypes were associated with multiple closely related variants. The spatial distribution of MAH strain genotypes provided little evidence of geographic structuring within the sampled population. Dominant strain genotypes were recovered from multiple birth and fattening farms across several cantons. None of the epidemiological clusters showed significant geographic clustering at either birth or fattening farm, however, larger epidemiological clusters tended to occupy a broader geographic area than smaller clusters. Although farms located within 20 km of one another were descriptively more likely to share identical strain genotypes and epidemiological clusters than more distant farms, this trend was not supported by permutation analyses, suggesting that geographic proximity alone explained only a limited proportion of MAH strain sharing. Together, these findings suggest that successful MAH strain genotypes are widely dispersed throughout the Swiss pig production system rather than being confined to specific regions. Such a pattern is compatible with the ubiquitous environmental distribution of MAH and the interconnected nature of Swiss pig production. However, the present study cannot distinguish between dissemination through animal movements and repeated exposure to widespread environmental reservoirs.

The temporal strain composition remained relatively stable throughout the study period, with several strain genotypes detected in both the retrospective 2015 dataset and the 2020–2022 samples. Of the 20 isolates analysed from 2015, 11 belonged to strain genotypes that were also identified in later years, whereas nine represented distinct genotypes that didn't reappear in the following sampling period. Together with the absence of major shifts in the geographic distribution of dominant strains over time, these findings suggest that certain MAH lineages are maintained within the Swiss pig production system over extended periods.

Longitudinal comparisons at the farm level revealed no identical strain genotypes among the four farms represented in both sampling periods. Most strain pairs differed by more than two alleles, suggesting changes in strain composition within individual farms over time. This pattern was observed even among pigs originating from the same recorded birth location, indicating that the temporal differences were not readily explained by animal origin. This finding may reflect the presence of multiple environmental sources of MAH exposure or temporal changes in environmental strain composition. However, the limited number of farms with longitudinal data precludes firm conclusions regarding strain persistence at the farm level.

At the farm level, most farms harboured multiple strain genotypes and epidemiological clusters, indicating a complex population structure that appears inconsistent with simple clonal spread. The coexistence of multiple strains within individual farms suggests repeated introductions, environmental exposure, or multiple transmission pathways. Given the ubiquitous nature of MAH and its ability to persist in soil, water and bedding material, exposure to multiple infection sources is the most plausible explanation[5, 26, 39, 40].

The widespread occurrence of diverse strain genotypes at both birth and fattening farms suggests that pigs may be exposed to MAH throughout the production chain, including early life. This observation is consistent with previous studies identifying environmental exposure as an important source of MAH infection in pigs[8, 33, 34, 41]. Although identical strain genotypes were occasionally recovered from 11 pigs originating from four birth locations, the site of infection could not be determined due to the study design. Additionally, four of these pigs each harboured two closely related strain pairs differing by only one or two alleles. This finding is compatible with microevolution from a common ancestor, which has been demonstrated for chronic *M. avium* infections, but could equally represent repeated infection with genetically related strains from environmental reservoirs [42, 43]. Overall, the predominance of more distantly related strain pairs supports repeated exposure to genetically diverse MAH populations rather than transmission of a single genotype throughout the production chain.

Strain diversity estimates strongly correlated with the number of sampled animals, indicating that sampling effort had a major influence on the observed diversity. Farms represented by more animals were more likely to contain a greater number of strain genotypes. Therefore, comparisons of diversity indices between farms should be interpreted cautiously.

A notable finding of this study was the complexity of infection patterns observed within individual pigs. Localized infections caused by a single strain, generalized infection involving the same strain in multiple tissues, and mixed infections within the same animal were all identified. Nearly half of all animals (56/117) harboured more than one strain genotype, with up to six distinct strain genotypes detected in a single animal, highlighting substantial within-host diversity. Nevertheless, the significant effect of animal identity indicated that isolates recovered from the same animal were generally more similar to each other than to isolates obtained from different animals. Co-occurring strain genotypes ranged from closely related to highly divergent, sometimes even within the same animal. Twenty-five of the 56 multi-strain pigs harboured at least one pair of strain genotypes differing by only one or two alleles, whereas 27 harboured at least one pair differing by five or more alleles. These findings provide clear evidence that multiple-strain infections in naturally infected pigs can arise through different epidemiological processes rather than a single infection event. Overall, these results demonstrate that analysing a single isolate per animal substantially underestimates within-host MAH diversity and highlight the importance of multi-tissue sampling to capture the full extent of strain diversity.

Therefore, these findings also have important methodological implications. Many epidemiological studies rely on a single isolate per animal to characterize strain diversity and transmission dynamics. The high frequency of mixed infections observed in the present study suggests that such approaches may underestimate the true diversity of MAH populations and overlook co-circulating strains within individual hosts. Future studies incorporating systematic multi-site sampling and whole-genome sequencing will be valuable for disentangling repeated exposure, co-infection, and within-host evolution.

The detection of identical strain genotypes in multiple tissues indicates that generalized MAH infections occur in a subset of pigs. In addition, multiple strain genotypes were occasionally detected within the same organ, demonstrating that mixed infections can exist even at the tissue level and previous infection

does not provide protection from further exposure.

No statistically significant association between strain genotype and dissemination potential was identified after correction for multiple testing. Although some strain genotypes such as STR183 (3/3), STR171 (3/4), STR85 (4/5) and STR223 (11/16) appeared to be more frequently associated with generalized infection, the small number of animals infected by individual strain genotypes limited more comprehensive investigation.

INMV profiles / One Health Implications

Comparison with the established INRA Nouzilly MIRU-VNTR (INMV) typing scheme demonstrated that the high-frequency strains identified in this study correspond to previously described INMV profiles, suggesting that major *M. avium* subsp. *hominissuis* (MAH) lineages are consistently detected across studies. However, the typing scheme used in the present study provided substantially higher discriminatory power, thereby offering improved resolution of strain diversity. This enhanced performance is attributed to both the inclusion of *hsp65* sequence data and the selection of more informative VNTR loci.

Several loci included in the INMV scheme (MIRU-3, MIRU-7, and MIRU-32) showed no discriminatory capacity in the present dataset and were likewise non-informative in the dataset of Scherrer et al., suggesting that their contribution to strain differentiation is limited in the Swiss MAH population. In contrast, the optimized genotyping scheme applied in the present study enabled greater differentiation, including the identification of allelic variants not represented in the INMV database, such as MIRU-47 alleles with non-integer repeat numbers.

Despite the overall concordance with INMV-defined lineages, the distribution of strain genotypes revealed that 16 INMV profiles accounted for 60 distinct VNTR profiles covering most isolates (n = 302), whereas 18 INMV profiles represented only 23 VNTR profiles comprising 40 isolates. This reduction in strain diversity from 84 VNTR-defined profiles to 34 INMV profiles highlights a substantial loss of discriminatory resolution, indicating that the INMV scheme is not able to dissect major underlying genetic diversity by grouping closely related but distinct strains.

Comparison with previously published data further demonstrated that two strain genotypes (STR30 and STR45) were identical to strain genotypes described by Scherrer et al. and Ichikawa et al., with matching VNTR profiles and identical *hsp65* sequence types. In total, 38 isolates from pigs, cattle, and humans across Switzerland, the Netherlands, and Japan shared identical allelic profiles, indicating a previously characterised and geographically widespread lineage. Additionally, several isolates differed by only one (n = 2) or two loci (n = 3), which may reflect microevolution within this lineage.

Taken together, these findings suggest that while the INMV scheme is effective for identifying major and widely distributed MAH lineages, it underestimates the true genetic diversity of the population. The enhanced resolution achieved in this study highlights the value of population adapted VNTR schemes for improving strain discrimination and enabled the identification of geographically widespread lineages shared among pigs, cattle, and humans, thereby providing a more detailed basis for investigating MAH epidemiology and transmission dynamics in a One Health context.

Study limitations

First, isolates were obtained from pigs presenting granulomatous lesions at slaughter and therefore do not necessarily represent the Swiss pig population. Consequently, no conclusions regarding prevalence can be drawn. Second, the number of animals originating from individual farms was limited, and sampling was opportunistic rather than systematic, reducing the probability of detecting the full strain diversity associated with a given farm. Third, because isolates were recovered from pigs at slaughter and retrospectively linked to their birth and fattening farms, the time and location of infection could not be determined. Finally, numerous strain genotypes were represented by only a small number of isolates, limiting any conclusion on dissemination patterns and epidemiological associations.

CONCLUSIONS

The MAH population was characterized by high genetic diversity, comprising numerous rare strains alongside a limited number of dominant, geographically widespread strain genotypes shared across farms and years. Across the population, farm, animal, and organ levels, the observed diversity patterns consistently support repeated environmental exposure to genetically diverse MAH strains as the principal driver of infection, while also indicating that occasional within-host microevolution contributes to strain diversity.

Declarations

Ethics approval and consent to participate.

Tissue samples were collected postmortem during routine meat inspection at a commercial slaughterhouse. No animals were slaughtered specifically for this study and no additional procedures were performed for research purposes. Under the Swiss Animal Welfare Act, no ethical approval or permit for animal experimentation was necessary.

Clinical Trial Registration

Clinical Trial Registration : Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are available within the article and its additional file. In addition, the representative *hsp65* nucleotide sequences have been deposited in GenBank under accession numbers QB037722–QB037724.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

E.H., L.S., L.N., U.F. and G.G. contributed to laboratory work and data acquisition. L.N. performed the laboratory-independent analyses, including molecular typing, bioinformatic and statistical analyses, prepared all figures, and drafted the manuscript. G.G. conceived and supervised the study, contributed to study design and data interpretation, and critically revised the manuscript. S.S. and R.S. provided resources and contributed to manuscript review and editing. All authors read and approved the final manuscript.

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Use of artificial intelligence.

ChatGPT (OpenAI) was used to assist with language editing and the development of R code. All analyses, results, interpretations, and manuscript content were verified by the authors, who take full responsibility for the published work.

Table 3

Tables 3 is available in the Supplementary Files section.

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Figures

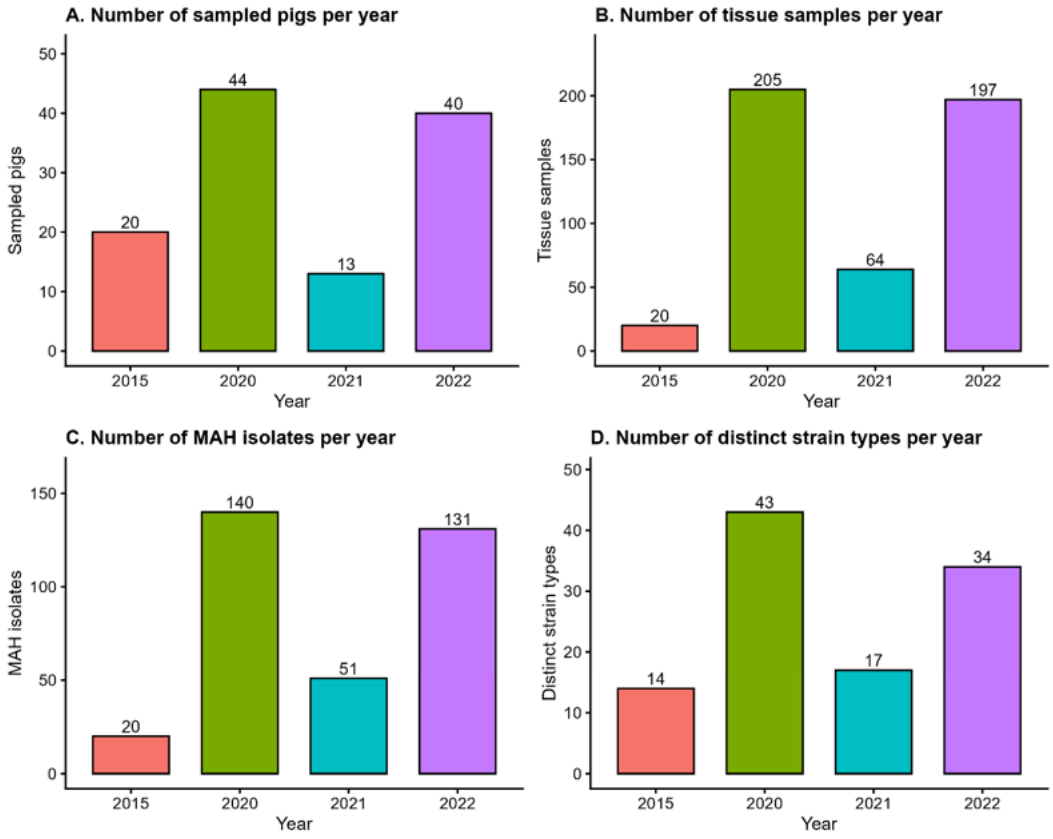


Figure 1

Temporal distribution by year of sampling. Shown are the number of sampled fattening pigs collected per year (A), number of tissue samples per year (B), number of isolates per year (C), and number distinct strain genotypes detected per year (D). Sample counts were based on unique sample IDs, whereas isolate counts included all cultured isolates, including subcultures derived from the same samples.

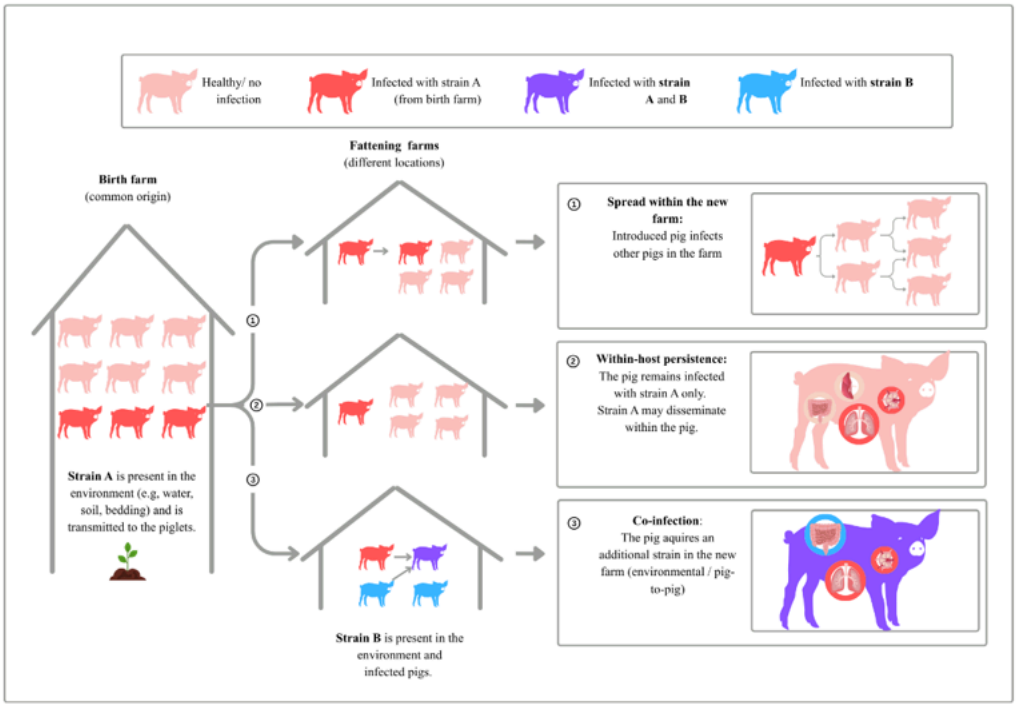


Figure 2

Conceptual model of potential transmission and dissemination pathways of *M. avium* subsp. *hominissuis* strain genotypes in Swiss pig production. Piglets originating from the same birth farm are initially exposed to strain A present in the farm environment. After transfer to different fattening farms, three possible scenarios are illustrated: (1) Spread within the fattening farm:strain A spreads to other pigs. (2) Within-host persistence:strain A persists and disseminates to multiple tissues within the pig. (3) Co-infection: the pig acquires an additional strain (strain B), resulting in co-infection.
Colors indicate infection status: light pink = uninfected; red = strain A; blue = strain B; purple = strains A and B.



Figure 3

Minimum spanning tree (MST) of the 84 *M. avium* subsp. *hominissuis* (MAH) strain genotypes based on combined VNTR allelic profiles and *hsp65* sequence data. Each node represents a strain genotype and is coloured according to the *hsp65* code. Node size is proportional to the number of isolates assigned to the respective strain. Numbers on connecting lines between nodes indicate allelic differences between strains.

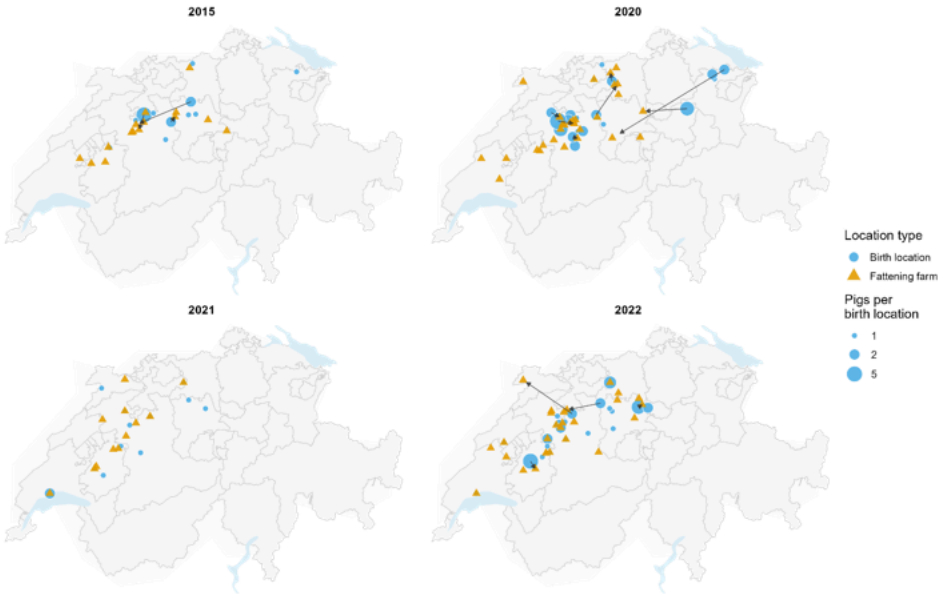


Figure 4

Spatial distribution of sampled pigs by year (2015, 2020, 2021, 2022). Birth locations are shown as blue circles and fattening locations as orange triangles: symbol size indicates the number of sampled pigs per location. Each point represents an individual location. Grey arrows indicate repeated birth to fattening farm movement involving more than one pig within the same sampling year. In total, 14 repeated movements were identified: 3 in 2015, 7 in 2020, 1 in 2021 and 6 in 2022.

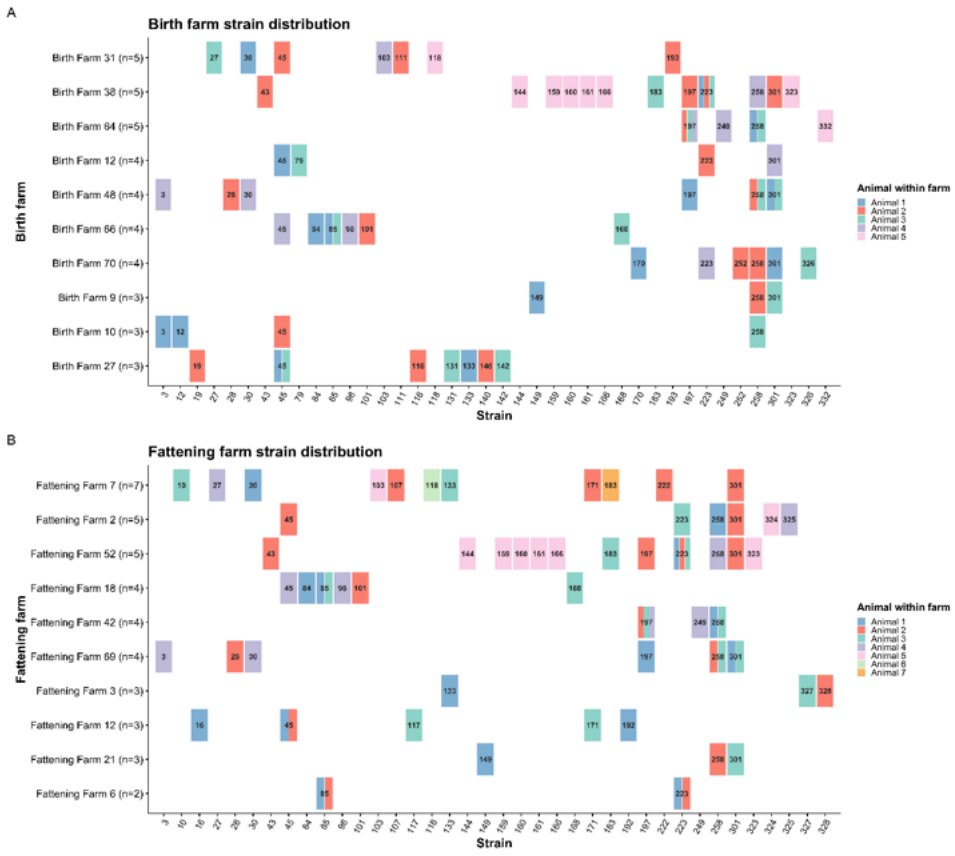


Figure 5

Distribution of *Mycobacterium avium* subsp. *hominissuis* genotypesamong birth (A) and fattening (B) farms. The 10 birth (A) and fattening (B) farms with the highest numbers of sampled animals are shown. Rows (y-axis) represent individual farms, and columns (x-axis) represent strain genotypes. The number of sampled animals from each farm is indicated in parentheses (n) next to the farm label. Colors distinguish individual animals within each farm and are used solely for visual differentiation. When the same strain genotype was detected in multiple animals from the same farm, the corresponding tile is subdivided proportionally to represent the number of positive animals. Numbers within tiles denote strain genotype IDs.

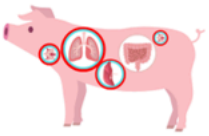
	Observation	Animals (n)	%	Illustration of distribution within animals
①	Single strain, single tissue	30	25.6	
②	Single strain, multiple tissues	31	26.5	
③	> 2 strain types within different tissues	35	29.9	
④	> 2 strain types within the same tissue	21	17.9	
	Maximum strain types per animal			6

Figure 6

Schematic patterns of *M. avium* subsp. *hominissuis* strain genotype distribution within pigs. The figure summarizes the number and proportion of pigs with single or multiple MAH strain genotypes detected across sampled tissues. Coloured markers represent different strain genotypes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.docx
- AdditionalFile2.docx
- AdditionalFile3.docx
- AdditionalFile4.docx
- Table3.docx