Paleopathological and Biomolecular Study of Tuberculosis in a Medieval Skeletal Collection From England

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ABSTRACT Nine human skeletons of medieval date from a rural English burial site show signs of skeletal tuberculosis. They were subject to polymerase chain reaction (PCR) assays aimed at detecting traces of DNA from infecting mycobacteria, with the purpose both of confirming the paleopathological diagnosis of tuberculosis and determining in individual cases whether disease was due to *M. tuberculosis* or *M. bovis*. In all nine cases, evidence for *M. tuberculosis* complex DNA was found, and in all instances it appeared that disease was due to *M. tuberculosis* rather than *M. bovis*. The significance of the findings for understanding tuberculous infection in rural agrarian communities in medieval England is discussed. Am J Phys Anthropol 114:298–311, 2001.

Tuberculosis is an infectious disease which may be acute or chronic, and which may affect any organ in the body, including the skeleton. The principal causative agents of tuberculous disease in humans are two bacteria of the *Mycobacterium tuberculosis* complex, *M. tuberculosis* and *M. bovis*. *Mycobacterium tuberculosis* is an acid-fast bacillus which has microbial and biochemical characteristics almost identical with *Mycobacterium bovis*, which is responsible for causing disease in diverse animal species, particularly cattle, as well as in humans (O'Reilly and Daborn, 1995).

Transmission of *M. tuberculosis* is generally by inhaled droplet infection from person to person, so disease caused by this microorganism generally begins as a respiratory infection. *M. bovis* may be acquired via inhalation of contaminated aerosol from live infected animals or carcasses, producing pulmonary disease. It may also be transmitted by consumption of contaminated milk or meat, in which case the primary lesion is located in the cervical lymph nodes (due to oropharangeal penetration by ingested bacteria) or the alimentary tract (Aufderheide and Rodriguez-Martin, 1998).

Skeletal tuberculosis is, almost without exception, a result of hematogenous spread of infection from soft-tissue foci. Skeletal disease only occurs in a minority of instances; data from the preantibiotic era indicate that about 5–7% of cases showed bone changes (Steinbock, 1976). The bony lesions permit the identification of tuberculosis in skeletal remains, and indeed abundant palaeopathological evidence for the disease has accumulated from around the world (e.g. Morse, 1967; Steinbock, 1976; Buikstra, 1981; Roberts and Manchester, 1995; Ortner, 1999).

Today, most human cases of tuberculosis are caused by *M. tuberculosis* rather than *M. bovis*, but the extent to which this applies to earlier populations is unclear. Because *M. tuberculosis* is spread person to person by droplet infection, it is a population density-dependent disease (Manchester, 1984). On the other hand, infection with *M. bovis* is favored by close human-animal contact. Before effective steps were taken in the mid-20th century to eliminate tuberculosis from cattle, the disease was rife in British herds (Collins and Grange, 1983), and the proportion of tuberculous disease in the human population due to *M. bovis* was much greater than it is today (Rich, 1951).

It has been suggested (Manchester, 1991) that in antiquity there was a baseline of *M. bovis* infection, upon which was superimposed *M. tuberculosis* disease. Although disease caused both by *M. tubercu-*

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losis and *M. bovis* is likely to have affected rural and urban populations in the past, the balance between the two may have differed in different communities, *M. tuberculosis* being favored in crowded urban centres, while small, scattered, agrarian communities may have harbored a greater proportion of cases due to *M. bovis*. *M. tuberculosis* disease may have risen in importance through time as a consequence of increasing urbanization and aggregation of population (Manchester, 1991).

From the point of view of tracing the history of tuberculosis, it is clearly of interest to investigate the balance between disease caused by the two bacilli in earlier human groups. Although disease due to *M. tuberculosis* and due to *M. bovis* may each affect the skeleton (Davies et al., 1984), it is impossible to distinguish the two forms of tuberculous disease using osteological criteria, as bony lesions caused by the two pathogens are anatomically similar (Ortner, 1999). However, this distinction is possible using ancient DNA. Analysis of human remains has demonstrated that fragments of *M. tuberculosis* complex DNA may survive over several millennia both in soft-tissue remnants (Salo et al., 1994; Nerlich et al., 1997; Pap et al., 1999), and in bone (Spiegelman and Lemma, 1993; Arriaza et al., 1995; Baron et al., 1996; Taylor et al., 1996; Baxarias et al., 1998; Braun et al., 1998; Faerman et al., 1999; Horáčková et al., 1999; Haas et al., 1999; Nuorala, 1999; Dutour et al., 1999) in remains of infected individuals. A variety of modern genomic techniques discriminate between members of the *M. tuberculosis* complex (Espinoza de los Monteros et al., 1998; Kamerbeek et al., 1997; Aranaz et al., 1996), and recent work (Taylor et al., 1999) has demonstrated the potential value of applying such techniques in paleopathological cases.

The purpose of the present work was to investigate tuberculous disease in an early cemetery sample. Likely cases of tuberculosis were identified using conventional paleopathological examination. Analyses of DNA extracted from these skeletons were then used, firstly to try to confirm the paleopathological diagnosis of tuberculosis, and secondly to attempt to determine, in individual cases, whether *M. tuberculosis* or *M. bovis* was the causative agent. In an attempt to investigate the presence of tuberculosis in the farmyard animals of this community, some animal bone fragments from the settlement associated with the cemetery, bearing bony signs of infection, were also analyzed.

MATERIALS

The skeletal material for this study comes from the rural agrarian site at Wharram Percy, England. Wharram Percy is a deserted medieval village, situated in the Yorkshire Wolds, an area of chalk uplands in northern England (Fig. 1). The site was subject to a long-running research excavation (Beresford and Hurst, 1990). An important focus of the fieldwork at Wharram Percy was St Martin's church and churchyard; excavations here resulted in the recovery of 687 articulated human skeletons. The burials, which date primarily from the 10th–16th century AD, are mainly of ordinary peasants who lived at the village of Wharram Percy or at other settlements and farmsteads in this rural parish. Several aspects of the Wharram Percy collection render it particularly suitable for present purposes. The chalk geology at the site has resulted in excellent macroscopic bone preservation, facilitating paleopathological study. Only those resident in the parish were accorded burial in the churchyard, so the skeletal material represents a geographically defined population. As well as the churchyard, large areas of the settlement have been subject to archaeological investigation. This provides additional background information concerning living conditions, aiding interpretation of the paleopathological data.

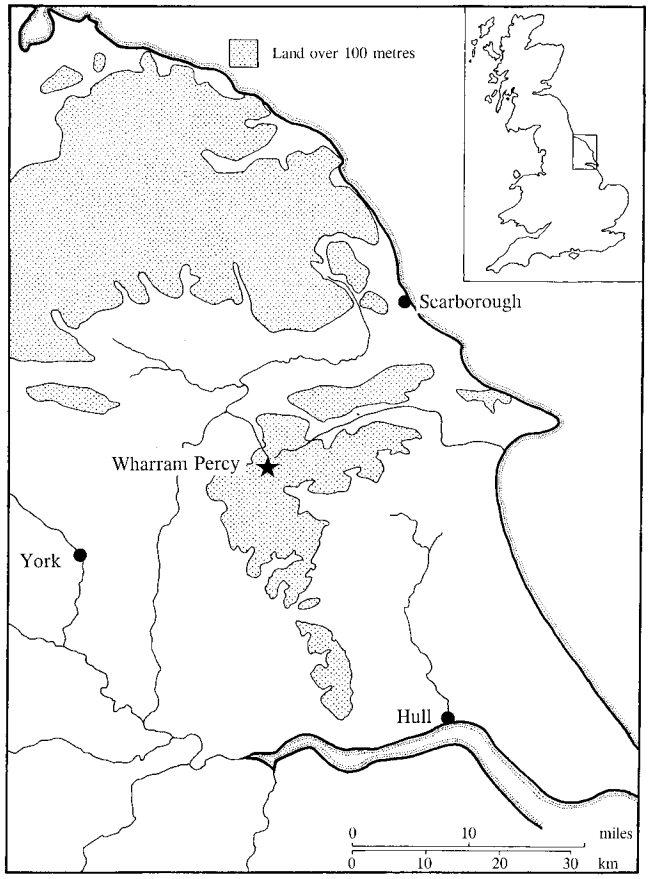

Fig. 1. Location of Wharram Percy.

OSTEOLOGICAL METHODS

All human burials from Wharram Percy have been examined osteologically by one of us (S.M.). Nine show signs of tuberculosis, and are the subject of the present work. Pathological changes were recorded using gross and radiographic examination. Tuberculosis was diagnosed according to the morphology of the lesions and their skeletal distribution.

Account was taken of the diagnostic criteria presented by Ortner and Putschar (1985), Steinbock (1976), Aufderheide and Rodriguez-Martin (1998), and Kelley and El-Najjar (1980). In brief, the more important of these include: predilection of lesions for the lumbar and thoracic vertebral bodies and the large joints of the appendicular skeleton; lesions that are mainly destructive, producing cavitation into the cancellous bone of the long-bone ends or vertebral bodies with little new bone formation; sequestra are characteristically absent; frequent vertebral body collapse with spinal kyphosis; and spinal disease is generally monofocal.

All nine burials showing evidence of tuberculosis are adult. Sex was established using dimorphic aspects of the pelvis and skull (Brothwell, 1981). Age at death was estimated using dental wear, calibrated using the juvenile part of the assemblage (Brothwell, 1981; Mays et al., 1995; Miles, 1963).

Study of faunal remains from Wharram Percy (Stevens, 1992; Pinter-Bellows, 1992) has indicated the presence of a number of cattle bone fragments showing signs of infection in the form of periostitis. Although tuberculosis in cattle may on occasion affect the bones (Lignereux and Peters, 1999), adequate paleopathological criteria for the diagnosis of the disease in faunal remains from archaeological sites have yet to be formulated. In cattle, as in man, chronic lesions in the lungs are a prominent feature of tuberculous disease (Hardie and Watson, 1992). In humans, it appears that chronic pulmonary tuberculosis may be associated with periosteal reactions on the ribs (Kelley and Micozzi, 1984; Roberts et al., 1994), so it seems reasonable to speculate that this may also be the case for cattle, although there is currently no evidence available to confirm this (Lignereux and Peters, 1999). Although rib periostitis cannot be considered diagnostic for pulmonary tuberculosis in cattle, periostitic bovine rib fragments probably form the most promising faunal remains available for biomolecular analysis as far as the present purposes are concerned. Three cattle rib fragments with periostitis were located in the Wharram Percy faunal collections; these were subjected to DNA analyses.

BIOMOLECULAR METHODS

DNA extracts were analyzed for the presence of *M. tuberculosis* complex. A number of further analyses were performed to discriminate between *M. tuberculosis* and *M. bovis*. Two human skeletons with no bony infectious lesions, and for which there were clear indications that death was unrelated to infectious disease (both individuals showed fatal cranial trauma), were used as controls. The DNA study was performed by G.M.T. in the Department of Medical Microbiology, Imperial College, London.

The skeletal lesions of brucellosis may resemble those of tuberculosis. Like tuberculosis, skeletal brucellosis shows a predilection for the vertebral bodies and large joints, and forms lytic lesions with little reactive bone except in the healing phase (Ortner and Putschar, 1985). The spread of brucellosis to human subjects depends upon contact with animal vectors, so those in agrarian communities like Wharram Percy might be considered to have been at risk. In consideration of the above, analysis for brucella DNA was also performed on the nine diseased human skeletons.

The samples for biomolecular analysis were taken from cancellous bone from the interior of a diseased element from each of the nine human suspected cases of tuberculosis. Cancellous bone tissue was also taken from the two human skeletons serving as controls. Bone samples, in the form of surface scrapings, were taken from the three bovine rib fragments showing periostitis. DNA was extracted from bone powder (100–200 mg) using a Nuclisens® guanidinium/silica kit (Organon Teknica, Boxtel, The Netherlands). A total of three extracts was prepared from all but one of the suspected tuberculosis cases; the exception was burial NA046, where only a single extract was prepared. A single extract was prepared from the bovine rib samples.

Polymerase chain reaction (PCR)

General PCR conditions, reagents, and thermal cyclers were common to all methods and have been described in detail elsewhere (Taylor et al., 1999). In heminested methods, 1 μl of first-round products was used to "spike" the second amplification step.

Measures were taken to avoid contamination, as described in detail elsewhere (Taylor et al., 1996, 1997). Briefly, bone extracts were prepared in a laboratory previously unused for tuberculosis research. Thereafter, a two-laboratory, three-workstation strategy was adopted so that PCR setup, amplification, and analysis of products were all performed in different areas. Autoclaved disposable plasticware, dedicated reagents, and pipettes with aerosol-resistant tips were used. Extraction and template blanks were included in every PCR assay, and were always free from product, indicating that contamination was well-controlled.

The procedures detailed below were carried out on the human bone samples. Two of these procedures, PCR for IS6110 and RD7, were also carried out on the three animal bone samples.

Mycobacterium tuberculosis *complex*. In an attempt to confirm the palaeopathological diagnosis of tuberculosis, PCR analyses for the RNA polymerase beta gene (*rpoB*) and the repetitive insertion element IS6110 were performed to detect *M. tuberculosis* complex.

rpoB. A pair of biotinylated primers was used to amplify a 157-base pair (bp) fragment of the RNA polymerase beta (*rpoB*) gene, accession number L27989. The sequence of the primers, and analysis of the product using the INNO-LiPA line probe assay to confirm *M. tuberculosis* complex, have been

described recently for other samples (Taylor et al., 1999).

IS6110. A nested PCR for the IS6110 insertion element was performed as previously described (Taylor et al., 1996). This is a modification of the method originally reported by Thierry et al. (1990).

Species-defining polymorphisms. The following procedures were carried out in an attempt to distinguish *M. tuberculosis* from *M. bovis*.

mtp40. PCR for mtp40 was performed as previously described (Taylor et al., 1999). The mtp40 element can be used to identify differences within the *M. tuberculosis* complex; it is present in the vast majority of *M. tuberculosis* isolates and absent from the great majority of *M. bovis* isolates (Liebana et al., 1996; Weil et al., 1996). Absence of mtp40 from *M. bovis* strains is associated with a deletion event, RD5, which is 8,964 bp in size and located between genome positions 2,626,067–2,635,031 on the H37Rv reference strain (Gordon et al., 1999).

oxyR. Primers were designed to amplify a fragment from the *oxyR* pseudogene of the *M. tuberculosis* H37Rv strain (accession number U16243). A heminested procedure was used. The sequence of the forward primer (F1) was 5′-CGCGCTGTCAGAGCTGACTTT-3′, and of the reverse primer (R1) 5′-TCTGCGGAATCAGTGTCACC-3′. Forty-three cycles of amplification were performed. Cycling conditions were 94°C for 20 sec, 66°C for 30 sec, and 72°C for 30 sec, generating a 150-bp product. In the heminested second round (127-bp product), primer R1 was used with an inner forward primer F2 of sequence 5′-TTGTGACTGCATGAGGGGC-3′. A further 35 cycles of amplification were performed. The stages were 94°C for 10 sec, 62°C for 30 sec, and 72°C for 15 sec. A final elongation cycle was performed at 72°C for 2 min. In some experiments, inner primers F2 and R1 were used for 43 cycles with 5 μl of bone extract to assess the requirement for heminested PCR.

pncA. The pyrazinamidase (*pncA*) gene is involved in susceptibility or resistance to the antituberculous drug pyrazinamide. A heminested PCR method was evaluated. In first-round amplification, a forward primer (F1) 5′-ATC-AGC-GAC-TAC-CTG-GCC-GA-3′ was used with reverse primer (R1) 5′-GAT-TGC-CGA-CGT-GTC-CAG-AC-3′ followed, in the heminested procedure, with F1 and a second reverse primer (R2) 5′-GGA-GTA-CCG-CTG-ACG-CAA-TG-3′. These result in a final product of 140 bp. Cycling conditions were 43 cycles at 66°C for 30 sec, 72°C for 20 sec, and 94°C for 20 sec, followed by one cycle at 72°C for 3 min. In the heminested stage, 35 cycles of amplification were performed as for first round, except that the annealing temperature was 65°C.

Spoligotyping. Spoligotyping (spacer oligotyping) provides simultaneous detection and typing of *M. tuberculosis* complex members. Spoligotyping relies on PCR amplification of variable sequences specifically found within the direct repeat (DR) region of the genome of *M. tuberculosis* complex members, in combination with their hybridization to a checkerboard of known variable elements or spacers (Kamerbeek et al., 1997). The DR consists of repeated sequences 36 bp long, interspersed by nonrepetitive DNA spacers (35–41 bp long). Hybridization patterns vary between isolates. *M. bovis* strains can be distinguished from *M. tuberculosis* by the absence of the terminal five spacers (nos. 39–43) at the 3′ end of the DR region. Spoligotyping was performed as described by Kamerbeek et al. (1997), with the modification of an increase in cycle number to 45 cycles.

***M. bovis*-specific fragments.** PCR for an *M. bovis*-specific fragment was performed using primers JB21 and JB22, as described by Rodriguez et al. (1995). Cycling parameters were modified to 45 cycles at 94°C for 25 sec, 62°C for 30 sec, and 72°C for 30 sec.

A second *M. bovis*-specific PCR was developed to amplify shorter fragments of DNA. The target was a length of DNA flanking deletion region 2 (RD2). The primers span a 12.7-kb fragment of the *M. tuberculosis* genome which is not present in *M. bovis* (Zumárraga et al., 1999a). This deletion is now known as RD7 and extends from bp 2,208,003–2,220,721 on the *M. tuberculosis* genome (Gordon et al., 1999). Under the PCR conditions used, no product is obtained from *M. tuberculosis*; in *M. bovis* the primers generate a band of 211 bp. The sequence of these was: (forward) 5′-ACTTCAGTGCTGGTTCGTGG-3′ and (reverse) 5′-ATCTTGCGGCCCAATGAATC-3′. Cycling conditions were 43 cycles at 66°C for 30 sec, 72°C for 20 sec, and 94°C for 20 sec, followed by one cycle at 72°C for 2 min.

Brucellosis. The target for amplification was insertion sequence IS6501, first reported in *Brucella ovis* (Ouahrani et al., 1993). This occurs in all strains of Brucella bacteria so far tested, and is present in variable copy number (5–35) depending on the species. A heminested strategy was adopted. The primer sequences were (F1) 5′-TAACCGATTATTTGTCGACGC-3′ and (R1) 5′-GCG-TGGACTTTCGATATGGT-3′. In the second round, primer F1 was used with the second reverse primer (R2) 5′-CATGGAAAAGCCTTTCCCAT-3′. Primers were synthesised on an Applied Biosystems model 381A DNA synthesiser. Amplification consisted of 35 cycles at 94°C for 40 sec, 60°C for 30 sec, and 72°C for 15 sec, followed by one cycle of 72°C for 2 min. The final product of 147 bp is situated from nucleotides 165–311 inclusive in accession no. X71024.

PCR minimum detection limits (Table 1)

Tuberculosis. The minimum detection limits for all the tuberculosis methods were established using

TABLE 1. Minimum detection limits of PCR methods applied to Wharram Percy cases[1]

PCR method	Product size (bp)	Cycles	Minimum detection
rpoB single round	157	43	150 fg
IS6110 nested	93	35 × 35	15 fg
mtp40 single round	152	43	1.5 pg
oxyR heminested	127	43 × 35	1.5 pg
oxyR inner primers	127	43	1.5 pg
M. bovis RD7	211	43	7.4 pg
pncA heminested	140	43 × 35	34 pg
Brucella heminested	147	35 × 35	34 pg

[1] bp, base pairs; fg, femtogram (i.e., 10^{-15} g); pg, picogram (i.e., 10^{-12} g).

serial dilutions of a stock extract containing 300 μg/ml of partially purified genomic DNA from the H37Rv strain of *M. tuberculosis*.

Sensitivity of the "in-house" *M. bovis* PCR for RD7 was similarly determined using serial dilutions of partially purified genomic DNA purified from heat-killed *M. bovis* strain AF 2122/97. A sensitivity of 10 femtograms has been reported for the *M. bovis*-specific PCR method of Rodriguez et al. (1995).

Brucella. *B. suis* DNA clone (clone 1330), containing 675 μg/ml DNA, was used. Serial dilutions of the clone in high-pressure liquid chromatography (HPLC)-grade water were prepared to establish the minimum detection limits of the PCR. A 10^{-2} dilution served as a positive control in all PCR reactions.

The most sensitive tests, the IS6110 and *rpoB* PCRs, were applied to the samples from the two human skeletons used as controls.

Automated DNA sequencing

Cycle sequencing of PCR products was performed on a Hybaid Touchdown with the ABI Dye Terminator Ready Reaction Kit (Perkin-Elmer Applied Biosystems), according to the manufacturer's protocol, with analysis on an ABI 310 Genetic Analyser.

Gel electrophoresis

Routine gel electrophoretic analysis of products was performed on either 2% or 3% w/v agarose gels. Products for sequencing were subsequently run on 0.8% w/v LMP agarose (Gibco BRL, Life Technologies). Bands were excised from the gel with a sterile scalpel blade and were purified using either the NucleiClean™ DNA isolation kit (Sigma-Aldrich) or the Geneclean II kit (Bio 101, CA).

RESULTS

Osteological analyses

The age at death and sex of the humans showing signs of tuberculosis, together with a summary of the locations of bone lesions, are shown in Table 2. Some of the pathological changes are illustrated in Figure 2a–e. The most common locations for lesions were the spine and the hip, although other bones are also affected, most frequently the ribs (Table 2). In all cases where ribs were involved, lesions were located near the proximal ends and represent extensions of vertebral foci of infection. No evidence for periostitis on the visceral surfaces of ribs, of the form described by Kelley and Micozzi (1984) and Roberts et al. (1994) as reflecting pulmonary infection, was found among the suspected tuberculosis cases.

Each of the three bovine rib fragments showed periostitis. One specimen (S95 Context 2432) showed woven bone on its visceral surface; the other two (S85 Context 22 and S95B Context 2053) showed a mixture of woven and partially remodelled bone deposits on their lateral surfaces.

Biomolecular analyses

Bone samples from suspected human and animal cases and controls were screened for the presence of DNA from infecting bacteria, using PCR assays directed towards a series of genetic loci with defined specificity patterns. The results are summarized in Table 3.

TABLE 2. Summary of osteological results[1]

Burial	Sex	Age	Date	Lesions		
				Spine	Hip	Other
EE056	M?	50+	900–1400 AD*	Yes (t, l)	No	Yes (ribs)
G438	F	25–35	1060–1170 AD*	Yes (t, l)	No	Yes (ulna, ribs, ilium)
G482	M	50+	1060–1170 AD*	Yes (l)	No	No
NA026	F	35–45	900–1400 AD*	Yes (t)	–	Yes (ribs, scapula)
NA046	M	50+	890–1170 AD	Yes (l)	No	No
NA112	M	50+	890–1160 AD	No	Yes	No
NA197	M	50+	900–1400 AD*	No	Yes	No
SA013	F	35–45	1270–1410 AD	Yes (t)	No	Yes (ribs)
WCO142	M	Adult	900–1700 AD*	Yes (l, s)	Yes	Yes (ilium, femora, tibiae fibula, metatarsal, calcanei, foot phalanx)

[1] Age, estimated age at death in years; Date, calibrated radiocarbon dates for individual burials (95% confidence limits), except * 95% confidence limits for dates of use of the part of the churchyard in which the burial was found (given where there is no date available on individual burial); Lesions, lower-case letters give location of lesions in spine: t, thoracic; l, lumbar; s, sacral; –, part of skeleton missing.

rpoB. A region of the mycobacterial *rpoB* gene was amplified by PCR, and the product was analyzed for the presence of specific oligonucleotide sequences using the INNO-LiPA line probe assay. A PCR product was obtained in all eight samples tested from suspected cases. Only five of these produced the *M. tuberculosis* complex pattern during subsequent hybridization (Fig. 4). PCR product from the other three samples hybridized to a single band on the INNO-LiPA test strips, a result similar to that obtained in the case of nontuberculous mycobacteria such as might be found in the environment. The two human control samples were negative for *rpoB*.

IS6110. All but two of the human suspected tuberculosis cases were positive for IS6110, a specific marker for the *M. tuberculosis* complex (Fig. 3). The three animal bone specimens were negative for IS6110, as were the two human control samples. The IS6110 element is present in multiple copies in the genome of most *M. tuberculosis* isolates and in one or several copies in *M. bovis*. While detection of IS6110 identifies the presence of a member of the *M. tuberculosis* complex, it does not therefore distinguish *M. tuberculosis* from *M. bovis*.

mtp40. Seven cases were positive for mtp40, suggesting the presence of *M. tuberculosis* rather than *M. bovis*.

oxyR. The sequence of the *oxyR* pseudogene has been found to differ at nucleotide 285. In *M. tuberculosis* isolates there is a guanine at this position, and in *M. bovis* an adenine (Sreevatsan et al., 1996). Amplification of the *oxyR* pseudogene was positive in six cases; in each there was a guanine at position 285, consistent with identification of *M. tuberculosis*.

pncA. The sequence of the *pncA* gene, encoding a pyrazinamidase enzyme, differs between *M. tuberculosis* (which is pyrazinamide-sensitive) and *M. bovis* (pyrazinamide-resistant) by a mutation at nucleotide 169. In *M. tuberculosis* strains base 169 is cytosine, whereas in *M. bovis* it is guanine (Scorpio and Zhang, 1996). Although a PCR reaction for *pncA* often generated bands at or near the expected size, sequencing was difficult due to double or multiple base-calling. This may have been due to amplification of related genes from contaminating environmental bacteria, and precluded the use of the *pncA* assay for discriminatory typing.

Spoligotyping. Three cases were successfully spoligotyped, generating patterns suggestive of *M. tuberculosis* in each case (Fig. 5). Burial NA112 was spoligotyped twice from the same extract, and good reproducibility of the pattern was observed. However, typing from two separate extracts from burial NA026 yielded patterns with several discrepancies. This could arise if different fragments of the DR region happened to be preserved in different parts of the bone sample, and raises a note of caution in interpretation of spoligotype patterns from ancient DNA.

M. bovis-*specific fragments.* Rodriguez et al. (1995) described a PCR reaction for *M. bovis*, using primers JB21 and JB22. This assay produced no bands of the expected size when applied to the human bone samples. However, this method is based on amplification of fragments of about 500 bp, and a negative result could reflect the extent of DNA degradation in the Wharram Percy material. Nevertheless, PCR for the shorter 211-bp fragment RD7 of *M. bovis* was also negative in all the Wharram Percy samples we examined, allowing some confidence that *M. bovis* was truly absent. Preliminary experiments confirmed that this method amplified *M. bovis* strain AF 2122/97, *M. bovis* BCG, and several clinical isolates causing disease, but failed to amplify the H37Rv and CDC1551 reference strains of *M. tuberculosis*. PCR for the RD7 fragment was also applied to the three animal bone samples, and in each case proved negative.

IS6501. All human samples were negative by heminested PCR for the insertion sequence IS6501, present in multicopy in all species of Brucella which produce disease in humans (Ouahrani et al., 1993). Preliminary experiments (not shown) confirmed that the assay detected various biovars of *B. suis*, *B. ovis*, *B. melitensis*, and *B. abortus*, as well as *B. neotomae*, *B. canis*, and *B. maris*.

In summary, for all nine of the suspected tuberculosis cases in the human skeletons, evidence from at least two distinct genetic loci allowed identification of the presence of DNA from organisms belonging to the *M. tuberculosis* complex. DNA from other related organisms was detected in some assays (e.g., the *rpoB* amplification) and is most probably a result of contamination of the samples with environmental bacteria. Each of the nine cases gave one or more positive results in assays designed to distinguish *M. tuberculosis* from *M. bovis*. In every case, the observed genotype resembled present-day *M. tuberculosis* rather than *M. bovis*. There was no evidence for Brucella infection.

The three animal bone specimens proved negative for the RD7 PCR, and for the analysis for IS6110, the most sensitive of the methods used in this work for detection of *M. tuberculosis* complex.

DISCUSSION

The biomolecular analyses confirmed the presence of *M. tuberculosis* complex in each of the nine humans in whom tuberculous disease was suspected on osteological grounds. This adds to a growing corpus of paleopathological cases in which osteological diagnosis of tuberculosis from dry bones has been supported by DNA evidence (e.g., Baron et al., 1996;

Fig. 2.

Taylor et al., 1996; Baxarias et al., 1998; Braun et al., 1998; Haas et al., 1999). The concordance between osteological and biomolecular results strengthens the notion that tuberculosis can be reliably diagnosed osteologically in ancient human skeletons, despite the fact that many conditions produce skeletal lesions which may, to varying degrees, resemble it (Buikstra and Cook, 1981, their Table 2; Aufderheide and Rodriguez-Martin, 1998).

In most instances, the osteological lesions clearly fulfilled the criteria for diagnosis of tuberculosis presented in the published literature. The exception to this was burial NA046, where the sole pathological lesions consisted of superficial cavitation on the body of the fourth lumbar vertebra (Fig. 2c) and a similar, smaller erosion on the fifth. Our favored diagnosis on osteological grounds was that these represented early paradiskal lesions of tuberculosis, on the basis of their morphological resemblance to lesions of this type identified in skeletal remains from documented cases of tuberculosis (cf. Kelley and El-Najjar, 1980, their Fig. 1). However, other

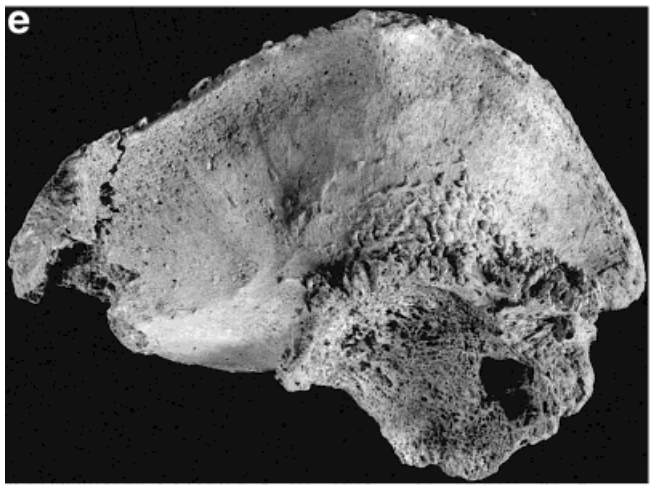

Fig. 2. Osseous lesions suggestive of tuberculosis in some of the Wharram Percy skeletons. **a:** Burial G438. Ninth thoracic through second lumbar vertebrae, showing destruction and collapse of vertebral bodies with spinal kyphosis. **b:** Burial SA013. Third through seventh thoracic vertebrae. T4–6 show erosions on the lateral/anterior aspects of their bodies. **c:** Burial NA046. Fourth lumbar vertebra, showing a small lytic lesion on the right side of the upper surface of its body. **d:** Burial SA013. Left sixth rib, showing lytic lesions at its head. **e:** Burial NA197. Right innominate, showing destruction of the acetabulum.

TABLE 3. Summary of biomolecular results[1]

Burial number	TB *rpoB* (2)	INNO LiPA (*rpoB* product) (1)	TB IS6110 (3)	TB mtp40 (3)	TB *oxyR* 285 (1)	DR region spoligotype (1)	*M. bovis* RD7 (1)	Brucella IS6501 (1)
Human suspected tuberculosis cases								
EE056	+(2)	*M. tb* complex	+(3)	+(2)	—	—	nd	−(1)
G438	+(2)	*M. tb* complex	+(3)	+(1)	+(G)	nd	−(1)	−(1)
G482	+(2)	Atypical pattern	+(3)	+(2)	+(G)	—	−(1)	−(1)
NA026	+(2)	*M. tb* complex	+(3)	−(2)	+(G)	*M. tb*	−(1)	−(1)
NA046	nd	nd	+(1)	−(1)	+(G)	*M. tb*	−(1)	−(1)
NA112	+(2)	Atypical pattern	−(3)	+(2)	+(G)	*M. tb*	nd	−(1)
NA197	+(2)	*M. tb* complex	+(3)	+(1)	—	—	−(1)	−(1)
SA013	+(2)	*M. tb* complex	−(3)	+(2)	+(G)	—	−(1)	−(1)
WCO142	+(2)	Atypical pattern	+(1)	+(1)	—	—	−(1)	−(1)
Control samples[2]								
EE003	−(1)	nd	−(1)	nd	nd	nd	nd	nd
G304	−(1)	nd	−(1)	nd	nd	nd	nd	nd
Animal bone samples[2]								
S85, Context 22	nd	nd	−(1)	nd	nd	nd	−(1)	nd
S95, Context 2432	nd	nd	−(1)	nd	nd	nd	−(1)	nd
S95B, Context 2053	nd	nd	−(1)	nd	nd	nd	−(1)	nd

[1] Numbers in parentheses at heads of columns are number of extracts tested by that PCR. Numbers in parentheses after results are numbers positive (+) or negative (−). *tb, tuberculosis;* (G), guanine; nd, not determined.
[2] One extract prepared.

diagnoses could not be confidently excluded on lesion morphology alone. For example, these lesions might also be consistent with other infections (e.g., brucellosis: Ortner and Putschar, 1985), malignant neoplastic disease (e.g., metastatic carcinoma: Kelley and El-Najjar, 1980), or trauma (Maat and Mastwijk, 2000).

Although the osteological indicators were ambiguous, NA046 was positive for three markers of the *M. tuberculosis* complex: IS6110, spoligotyping, and *oxyR*. The results from the latter two analyses indicated the presence of *M. tuberculosis* rather than *M. bovis*. The lack of an mtp40 PCR product in this instance may simply reflect the lower sensitivity of

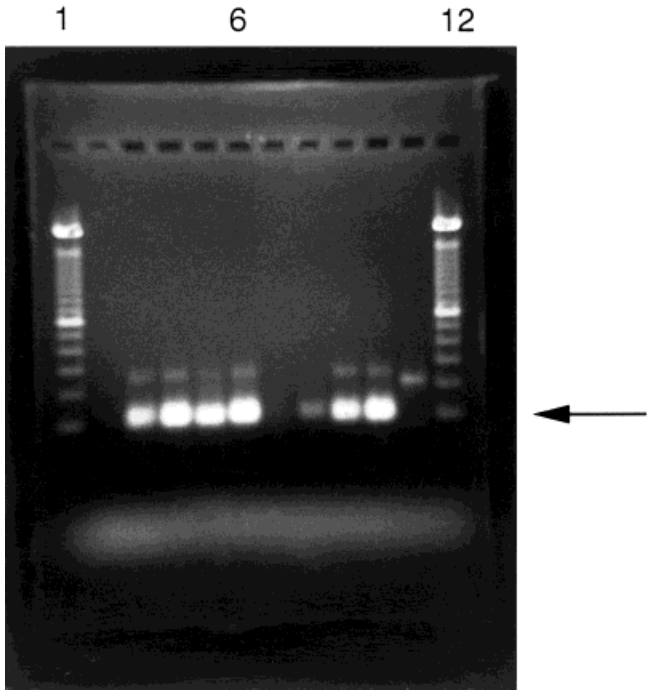

Fig. 3. IS6110 PCR. Gel electrophoretic analysis of nPCR products of 2% agarose gel. **Lanes 1** and **12**, DNA 100-bp ladders. **Lane 2**, water blank. **Lane 3**, H37Rv positive control. **Lane 4**, burial G438. **Lane 5**, burial NA026. **Lane 6**, burial WCO142. **Lane 7**, burial NA112. **Lane 8**, burial G482. **Lane 9**, burial NA197. **Lane 10**, burial EE056. **Lane 11**, burial SA013. Arrow denotes IS6110 PCR product.

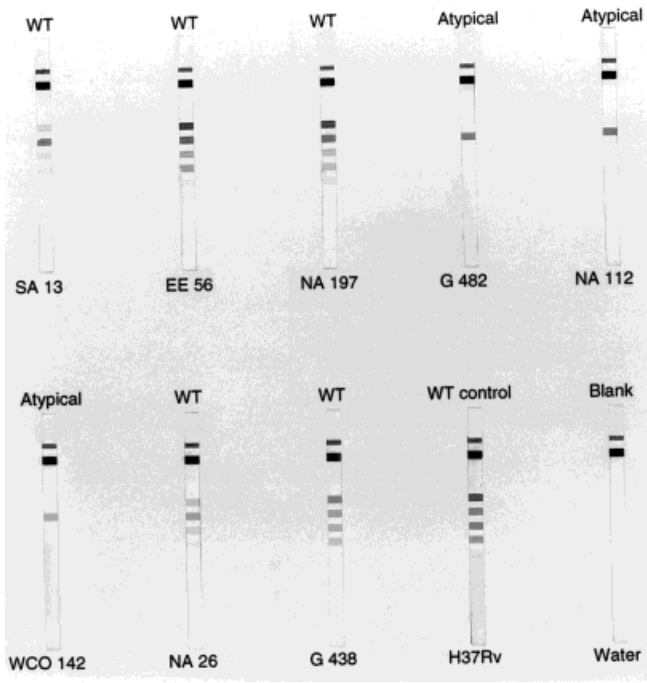

Fig. 4. *rpoB* PCR. Analysis of *rpoB* PCR products from eight of the suspected tuberculosis cases, using in INNO LiPA rif.TB kit. Five cases show typical wild-type (WT) pattern, indicative of rifampicin-sensitive *M. tuberculosis* complex strains (hybridization to probes S1–S5), and three show an atypical pattern, with hybridization only to probe S2.

this method compared to IS6110 (Table 1). Although the biomolecular results mean that we can be confident that this case was positive for *M. tuberculosis*, it is possible, given that the great majority of tuberculosis cases show no bony signs (Steinbock, 1976), that NA046 had tuberculous infection which had not affected the skeleton and that the spinal lesions simply reflect some unrelated, coincidental condition. However, given their morphological resemblance to lesions in documented cases of tuberculosis, a more parsimonious explanation is that the spinal changes are indeed early tuberculous lesions, and that the DNA analyses in this instance enabled a firm diagnosis which would otherwise have remained elusive.

Our experience with several different PCR methods on the Wharram Percy human bone material highlights three general points relating to the analysis of ancient DNA. Firstly, it confirms that it is advantageous to use methods that are targeted to amplify shorter fragments, which are likely to survive in ancient material. Secondly, the observation that not all PCRs were successful in each specimen indicates the value of applying several PCR techniques and of repeat sampling. Finally, it is important to be able to detect pathogen DNA in mixed samples containing contaminating soil microorganisms; this proved problematic in the case of *rpoB* and *pncA* assays in the present study.

Severe diagenesis, of the type thought to be caused by soil-dwelling microorganisms, has consistently been found by one of us (G.T.-W.) in histological sections of medieval human bone from Wharram Percy (Fig. 6). Severe alteration of bone by microbiological attack is typical of sites like Wharram Percy, which lie on calcareous geology (Millard and Hedges, 1995). Although it has been suggested (Hagelberg et al., 1991; Colson et al., 1997) that there may be a correlation between DNA survival and the quality of the bone microstructure, the present results demonstrate the potential for analysis of ancient pathogen DNA to produce useful paleopathological data, even in bone where preservation at the histological level is poor. This may be consistent with the idea that the tough cell wall characteristic of mycobacteria aids survival of their DNA during the decay process (Spiegelman and Donoghue, 1999).

All nine tuberculosis cases from the Wharram Percy churchyard appear to represent infection with *M. tuberculosis* rather than *M. bovis*. The only other study of which we are aware which has successfully distinguished *M. tuberculosis* and *M. bovis* disease in ancient human remains is an earlier report from our group (Taylor et al., 1999), in which two British medieval cases of tuberculosis were studied and, as in the present work, *M. tuberculosis* rather than *M. bovis* was found to be the causative agent. However, in contrast to the present study, those burials came from a cemetery from a large, crowded urban center,

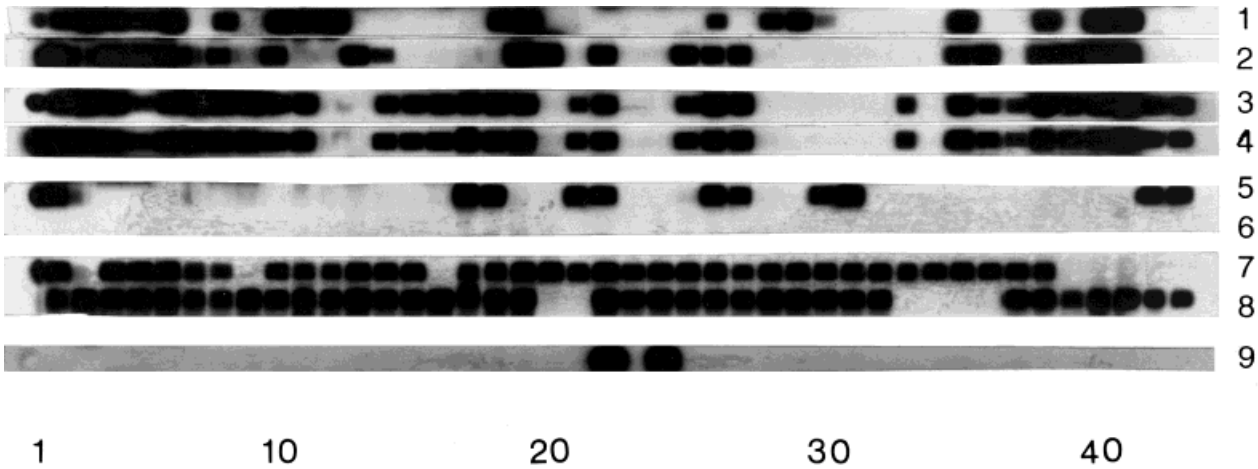

Fig. 5. Spoligotyping. Hybridization analysis of the DR region of *M. tuberculosis* complex organisms amplified from three Wharram Percy cases and controls. Rows 1 and 2, burial NA026 analyzed from two different extracts. Rows 3 and 4, burial NA112 analyzed twice from the same extract. Row 5, burial NA046. Row 6, water blank. Row 7, *M. bovis* BCG control. Row 8, *M. tuberculosis* H37Rv strain. Row 9, soil extract. The numbers along the bottom are spacer numbers.

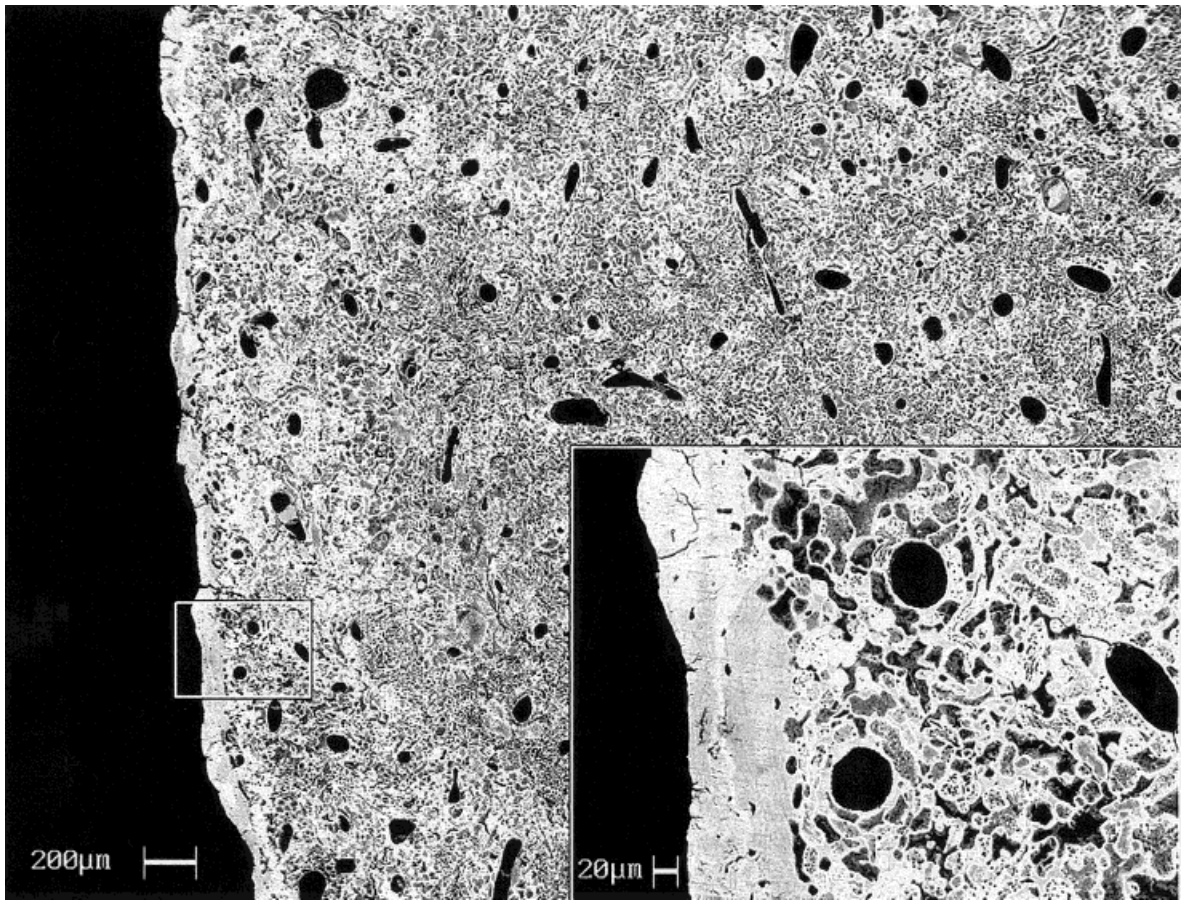

Fig. 6. Scanning electron micrographs from bone from a Wharram Percy burial. Although the outer 100 μm of bone preserve some histological detail, across most of the section advanced diagenesis is apparent. The great majority of histological structures have been destroyed or obscured by postmortem alteration of bone tissue, so that in general only gross features such as Haversian canals remain visible. The severe diagenetic changes seen in this specimen are typical of those in medieval human bone from Wharram Percy.

where it was no surprise to confirm the presence of *M. tuberculosis*.

Analysis of historical samples of *M. bovis* DNA will be of particular interest in the context of the rapidly expanding knowledge generated by characterization of contemporary mycobacterial genomes. While it is common speculation that *M. tuberculosis* adapted for human infection following transfer from domesticated livestock, recent characterization of genomic deletion events casts doubt on this scenario

and suggests that it is *M. bovis* rather than *M. tuberculosis* which has undergone more extensive adaptation (Behr et al., 1999; Gordon et al., 1999). The time-scale for these deletion events is unknown; the possibility should at least be considered that medieval *M. bovis* might have more closely resembled contemporary *M. tuberculosis* or a common progenitor of the *M. tuberculosis* complex. It is known from studies of contemporary *M. bovis* isolates that some do share molecular markers more commonly associated with *M. tuberculosis* strains. Examples include *M. bovis* isolates retaining the mtp40 element (Weil et al., 1996) and isolates from sealions and fur seals with *pncA* and *oxyR* polymorphisms usually associated with *M. tuberculosis* (Zumárraga et al., 1999b). Due to the variety of methods which have been used in the present study to differentiate *M. bovis*, it seems unlikely that this possibility could explain our findings at Wharram Percy. Further, all isolates of *M. bovis* we have previously examined, including those from fur seal, have tested PCR-positive for RD7 (Taylor, unpublished observations). This implies erasure of the 12.7-kb fragment downstream from *M. bovis* deletion region 2 (RD2) (Zumárraga et al., 1999a). Deletion of this 12.7-kb fragment from the *M. bovis* genome may have been an early event in the evolution of the *M. tuberculosis* complex.

At Wharram Percy it is clear that cattle formed an important part of the site's economy. Analyses of animal bones recovered from the settlement (Ryder, 1974; Stevens, 1992; Pinter-Bellows, 1992) indicate that sheep formed about 40–60% of the faunal remains, and cattle about 30–40%, with pigs 11% or less. Cattle were likely the most important source of meat. Finds of the remains of pottery vessels which would have been used for dairy products attest to the inclusion of these foods in diets (Beresford and Hurst, 1990). It was usual in the medieval period for humans and livestock to share the same living space (Lamond, 1890), and at Wharram Percy, excavations have uncovered the remains of peasant long-houses (Beresford and Hurst, 1990) which would have housed both human and bovine inhabitants, cattle being stalled at one end while the family lived at the other (Fig. 7).

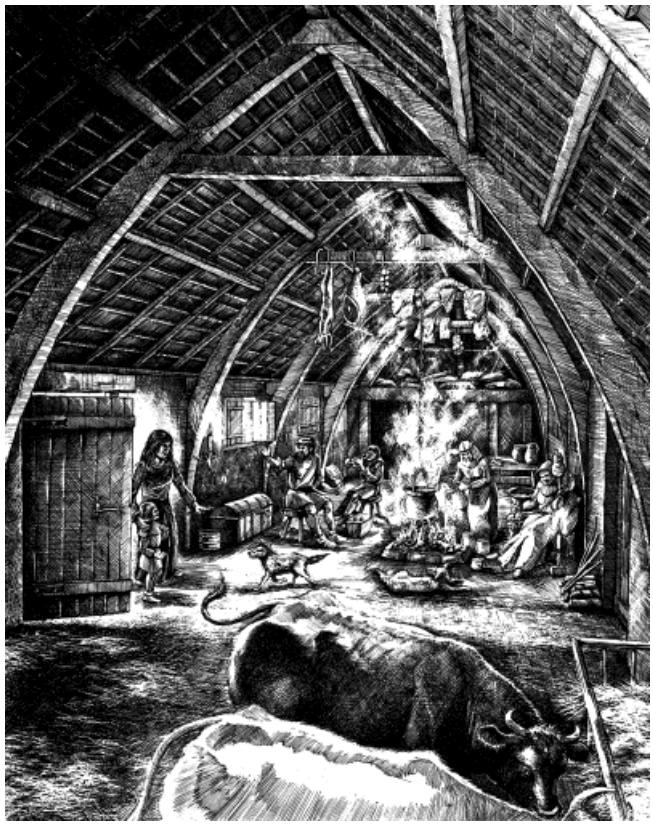

Fig. 7. Reconstruction of a peasant long-house at Wharram Percy. These dwellings housed both human and bovine inhabitants. ©English Heritage Photo Library.

In a community like Wharram Percy, where people had close contact with cattle, and where beef and dairy products formed important parts of the diet, it might be expected that there would have been significant risk of human infection from *M. bovis*. It is therefore perhaps surprising that all cases of tuberculosis identified here appear to have been caused not by *M. bovis* but by *M. tuberculosis*.

One possible explanation for the absence of *M. bovis* disease is simply that the Wharram Percy cattle may have been free of tuberculosis. Despite the evidence that prior to the steps taken to eliminate tuberculosis in cattle in the 20th century it was rife in British herds (Collins and Grange, 1983), it cannot be stated with certainty that it was present in cattle in medieval times. The three bovine rib samples were analyzed in an attempt to determine whether there was any evidence for tuberculosis among Wharram Percy cattle. That results proved negative may reflect poor DNA survival or that the periostitis shown by these specimens was due to some other disease. The results obtained do not therefore allow us to comment on whether or not tuberculosis was present in domestic stock at Wharram Percy.

If it is accepted that domestic stock were not the main source of tuberculosis among the human inhabitants at Wharram Percy, alternative models for infection from human sources need to be considered. As was stated above, spread of pulmonary tuberculosis is favored by high population densities. The population density of the parish served by Wharram Percy church is difficult to determine precisely, and varied greatly during the medieval period. Nevertheless, estimates of approximately 1–6 persons per square kilometer (calculated from figures presented in Bell, 1987), with a maximum reached during the late 13th–early 14th century, are probably not too wide of the mark. This is in line with population density figures estimated for Yorkshire as a whole in the medieval period (Smith, 1988). These figures are indicative of a sparsely populated area by medieval standards (Smith, 1998).

Rural Yorkshire would not appear to have provided an ideal environment for a population density-dependent disease such as pulmonary tuberculosis. Nevertheless, it does appear that tuberculosis can be maintained even in scattered populations (Black, 1975), so it might be suggested that the human form of the disease was endemic among rural communities in this area in the medieval period. However, the degree to which tuberculosis can be maintained in human groups of small size in the absence of an extrinsic reservoir of relatively large size is unclear (Buikstra and Cook, 1981). Population aggregates in urban centers may act as reservoirs of tuberculous infection, which may then "pulse out" to small rural communities in their hinterlands (Buikstra and Cook, 1981). Wharram Percy lies about 20 miles from York, which, with a population of about 12,000 in the medieval period (Goldberg, 1986), was easily the largest regional center, and was indeed one of the largest cities in England. Excavated human remains from York showing tuberculous lesions (Stroud and Kemp, 1993) confirm the presence of the disease there in medieval times.

In the medieval period, populations in urban settlements were maintained by immigration from surrounding rural areas. Russell (1948) offers documentary evidence that Wharram Percy would have lain well within the hinterland for migration of individuals to become resident in York, and analysis of skeletal demographic data (Mays, 1997a) suggests emigration from Wharram Percy to urban centers such as York. Ceramic and other artifactual evidence from Wharram Percy indicates trade with local towns such as Malton (Hurst, 1984). At Wharram Percy, a few remains of marine fauna (Ryder, 1974) have been recovered, and consistent with this, carbon stable isotope determinations (Mays, 1997b) have shown that marine foods were a significant source of protein in human diets here. Marine foods may have been obtained direct from fishing ports, such as Scarborough or Hull, or indirectly from trade with inland market towns.

The above serves to remind us that, despite its rural location, Wharram Percy was not an isolated community, but rather had a network of social and economic links with a range of other settlements. Perhaps contact with reservoirs of the disease in larger population centers may have helped maintain tuberculosis in the countryside. If the Wharram Percy results are anything to go by, it may be that models such as this, which invoke person-to-person transmission of disease, are more appropriate for understanding rural tuberculosis in the medieval period than reference to local animal vectors.

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