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Mycobacterium avium subspecies *paratuberculosis* in the inflamed gut tissues of patients with Crohn's disease in China and its potential relationship to the consumption of cow's milk: a preliminary study

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Summary

Mycobacterium avium subspecies *paratuberculosis* infection in domestic livestock is widespread in many countries throughout the world. Studies in Europe and the USA show that *M. avium* subspecies *paratuberculosis* can be cultured from retail pasteurized cow's milk and that these organisms are being transmitted to humans by this route. Most people with chronic inflammation of the intestine of the Crohn's disease type are infected with these chronic enteric pathogens. The production and consumption of cow's milk has increased in China and so also has the incidence of Crohn's disease. The present preliminary investigation was carried out to determine whether *M. avium* subspecies *paratuberculosis* is present in the intestinal tissues of Chinese patients with Crohn's disease who have never left China. Archival paraffin-embedded surgical pathology blocks from patients having surgery for Crohn's disease (CD) or for cancer (nIBD) in China were studied. *M. avium* subspecies *paratuberculosis* was detected by nested IS*900* PCR with Southern blotting and amplicon sequencing. The intestinal tissues of 9 of 13 (69.2%) CD patients and 2 of 14 (14.3%) nIBD patients were IS*900* PCR positive ($P = 0.0063$; odds ratio = 13.5). These initial studies suggest that people in China are exposed to *M. avium* subspecies *paratuberculosis* and that as in other countries, the infection is significantly associated with Crohn's disease. *M. avium* subspecies *paratuberculosis* in dairy herds and retail milk in China needs to be investigated.

Introduction

Crohn's disease (CD) in humans is a systemic disorder whose principal clinicopathological manifestation is chronic granulomatous inflammation of the intestine. It was first described by Dalziel (1913). It is a new disease which emerged during the twentieth century particularly in industrialized countries and has continued to increase in incidence and prevalence (Hildebrand *et al.* 2003; Phavichitr *et al.* 2003; Loftus 2004; Molinie *et al.* 2004). Recent evidence suggests that the incidence of CD is now rising in other regions of the world such as South America, India and Japan which were formerly thought to be low-incidence areas (Hermon-Taylor & El-Zaatari 2004).

Mycobacterium avium subspecies *paratuberculosis* is a member of the *M. avium* complex (Thorel *et al.* 1990; Gerlach 2002; Chacon *et al.* 2004). It is a chronic enteric pathogen with the specific ability to initiate and maintain chronic inflammation of the intestine of a range of histopathological types in many species including primates (Riemann & Abbas 1983; Chiodini *et al.* 1984; Clarke 1997). Despite this, *M. avium* subspecies *paratuberculosis* can live in animals for years without causing clinical disease. Subclinically infected animals secrete *M. avium* subspecies *paratuberculosis* in their milk (Sweeney *et al.* 1992). It is more thermotolerant than *M. bovis* (Sung & Collins 1998) and live *M. avium* subspecies *paratuberculosis* has so far been cultured from retail pasteurized milk supplies in the UK, Czech Republic and USA (Millar *et al.* 1996; Grant *et al.* 2002; Wuhib *et al.* 2004; http://www.johnes.org). *M. avium* subspecies *paratuberculosis* in humans is in a robust Ziehl–Neelsen (ZN)-staining-negative form which is

difficult to detect (Hermon-Taylor & El-Zaatari 2004). Research from several laboratories has confirmed that when optimized methods are used, the majority of people with CD are found to be infected with these chronic enteric pathogens (Schwartz *et al*. 2000; Bull *et al*. 2003; Naser *et al*. 2004; Romero *et al*. 2005).

Over the last 20 years the production (http://www.stats.gov.cn, http://www.dac.com.cn) and consumption (Yu *et al*. 2001; Du *et al*. 2004; Fraser 2004; Liu *et al*. 2004; Zhang *et al*. 2004) of cow's milk has substantially increased in China where the incidence of CD is also rising (Sung *et al*. 1994; Leong *et al*. 2004; Zheng *et al*. 2004). The present preliminary study on a limited number of subjects was carried out to determine whether *M. avium* subspecies *paratuberculosis* infection could be detected in the chronically inflamed intestinal tissues of Chinese patients having surgery for CD in China.

Materials and methods

Patient samples

Thirty coded archival paraffin blocks containing resected full thickness intestinal tissues from 27 Chinese patients, who underwent surgery at the Drum Tower Hospital, Nanjing University Medical School, China from 1996 to 2000, were studied. The diagnosis of CD was established on the basis of classical clinical, radiological, endoscopic, and histopathological criteria. Control subjects were all patients with colon cancer not known to have Irritable Bowel Syndrome. All samples were processed and tested blind.

Preparation of sample DNA

An optimized procedure for sample processing and DNA extraction was used which ensured access to MAP DNA. Removal of paraffin from pathology blocks was based on the method of Fredericks & Relman (1999). Briefly, three 25 μm sections were cut from each block. The microtome was cleaned with xylene between samples and a new knife was used for each block. The three sections were placed together in a 1.5 ml reaction tube. Tubes were coded and processed blind. To each tube was added 1 ml of *n*-octane (Sigma, Poole, UK), vortexed for 20 s at room temperature then 300 μl of methanol was then added and the tube again vortexed for 20 s. After centrifugation at 7000 $\times$ *g* for 2 min, the supernatant was removed and the tissue dried for 2 h under vacuum (Maxi Dry-Lo, Denmark). 600μ l of lysis buffer was added [10 mM Tris HCl pH 8.0, 2 mM EDTA, 400 mM NaCl, 0.6% SDS containing protease K 33 μ g/ml (Sigma)] and incubated overnight with shaking for 18 h at 37 °C. The sample was then transferred to a Lysing Matrix B Ribolyser tube (Qbiogene, Nottingham, UK; Cat N. 6911-100) and subjected to mechanical disruption at 6.5 m/s for 45 s. After placing the tube on ice for 15 min, the DNA was extracted using a standard phenol/chloroform procedure and the extract dried and re-suspended in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA buffer, pH 8.0).

Nested IS900 PCR

Nested IS*900* PCR using L/AV primers was performed in duplicate on each DNA extract as previously described (Bull *et al*. 2003). Briefly, 5 μl of DNA extract was added to PCR pre-mix containing primers 5′-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3′ with 5′-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3′ for the first round, and 5′-ATG TGG TTG CTG TGT TGG ATG G-3′ with 5′-CCG CCG CAT CAA CTC CAG-3′ for the second round, producing a final amplification product of 298 bp. A 50 μl PCR reaction mix contained 1 $\times$ PCR buffer (*Taq* Buffer B, Promega, Southampton, UK), 2 μM of each primer, 100 μM dNTP, 2.5 mM $MgCl_2$(1.5 mM $MgCl_2$ for second round) 2 units of Expand High Fidelity *Taq* polymerase (Roche, Lewes, UK). Cycle conditions were 94 °C for 5 min followed by 30 cycles first round of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 3 min, with a final extension of 72 °C for 7 min. The second round nested reaction was then performed using 2 μl of the first round product and the conditions indicated above with 30 cycles. The nested IS*900* PCR amplification products were visualized in 1.5% agarose gels stained with ethidium bromide. Each batch was run with process control and reagent control tubes, a positive control tube with 1–10 copies of plasmid pIDL60 containing IS*900*, and the stringent precautions previously described (Bull *et al*. 2003) to exclude and monitor the exclusion of contamination artifact.

Southern hybridization

Amplified products were electrophoresed on agarose gels and transferred onto positively charged nylon membranes. After transfer, the membranes were pre-hybridized overnight at 37 °C with pre-hybridization buffer (5 ml formamide, 2 ml 20× SSC, 2.5 ml 10% SDS, 0.5 ml H_2O with 50 mg powdered milk) and 100 μl salmon sperm DNA (10 mg/ml). The membranes were hybridized with 100 ng of a ^{32}P-labelled 298 bp IS*900* probe and denatured salmon sperm DNA (100 μg/ml) overnight at 37 °C. Finally the membranes were washed twice in stringency wash buffer (10 ml 20× SSC, 1 ml 10% SDS, 89 ml H_2O) for 20 min, and exposed to radiographic film at 70 °C for 72 h.

Amplicon sequencing

Five microlitres of original DNA extract from two CD patients previously shown to be IS*900* PCR positive were re-amplified using the same IS*900* PCR conditions as above but with 3.5 U of *Taq* polymerase (Expand High Fidelity PCR System, Roche). Amplicons of the expected size (298 bp) from the second round AV1/AV2 primers were excised and purified using QIAquick gel

extraction kit (Qiagen, Crawley, UK). Eight microlitres DNA was ligated into pGEM-T easy vector (Promega), transformed into DH5α competent cells (Invitrogen, Paisley, UK) and recombinants selected. Plasmid extractions (Qiagen) from two individual clones were then sequenced in both directions using second round nested primers (ABI PRISM 7700 at the Advanced Biotechnology Centre, Imperial college, London, UK. Sequence analysis DYNASTAR).

Statistical analysis

Fischer's exact test and odds ratios were used to assess the significance of apparent differences between CD and nIBD groups (SPSS Graduate Pack 11.5 for Windows, SPSS Inc., Chicago, IL, USA).

Results

The 13 Chinese patients with CD included 12 men and 1 woman with a median age of 40 years (range 19–66). The 14 nIBD patients having operations for cancer included 8 men and 6 women with median age 60.5 years (range 36–75). All 27 people lived in the region of Nanjing and had never left China. All specimens were formalin-fixed paraffin-embedded surgical samples of full thickness intestinal tissue. In the CD group, there were 16 pathology blocks from the 13 CD patients, 3 of whom each had 2 blocks. Seven of the 16 blocks contained ileum only, 2 contained ileum and colon, and 7 contained colon only. In the nIBD group, 1 block contained ileum only, 3 contained ileum and colon, and 10 were colon only. Nine of 13 CD patients (69.2%) and 2 of 14 nIBD patients aged 69 and 75 years (14.3%) were IS*900* PCR positive (Figure 1a and b) for *M. avium* subspecies *paratuberculosis* ($P = 0.0063$, odds ratio = 13.5). Positive tissues within the CD group comprised a total of 11 samples including 4 of small gut, 5 of colon and 2 of both. Positive tissues within the nIBD group were 1 of small gut and 1 of colon. All negative PCR and process controls reported negative. All positive control reactions reported positive. In 2 positive CD patients IS*900* AV1/AV2 amplicon sequences were 99.3% homologous to IS*900* GenBank sequence X16293 with the exception of consistent base substitutions CG → GC involving nucleotides 44 and 45 and G → A at nucleotide 125 of the amplicons. The primers and PCR conditions used in this study did not report related members of the IS*900* family of elements including IS*1613* (AJ011837), IS*1626* (AF071067) and *Mycobacterium* sp. V1 (AJ289786).

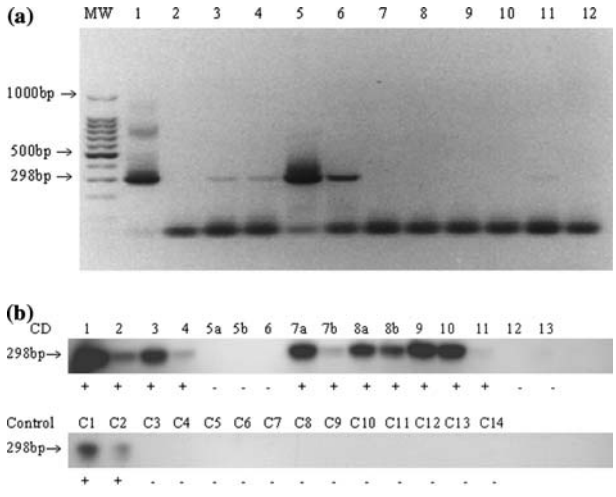

Figure 1. (a) PCR amplification products from CD and nIBD patients in China. MW-Molecular weight ladder (100 bp size), Lane 1 – TE buffer spiked with 50 copies pIDL60 plasmid containing IS900, Lanes 2–6–samples from CD, Lanes 7–11 –samples from nIBD intestine, Lane 12 –negative reagent control. (b) Southern blot confirmation of IS900 amplicons from the 13 CD and 14 nIBD patients.

Discussion

The robust ZN-staining-negative form of *M. avium* subspecies *paratuberculosis* present in low abundance in human intestine is difficult to detect (Hermon-Taylor *et al.* 2000). Archival formalin-fixed paraffin-embedded tissues, although convenient for screening studies, are not ideal, as the tissues are variably cross-linked and the target DNA may be fragmented. The detection rate of *M. avium* subspecies *paratuberculosis* in 69% of CD tissues in the present study is highly significant and is in agreement with previous research on Chinese patients carried out in the Chengdu region in the West of China (Gan *et al.* 1999). A causative relationship between *M. avium* subspecies *paratuberculosis* and Crohn's disease, although still controversial, is increasing in recognition (Greenstein & Collins 2004). The present study provides further evidence implicating these chronic enteric pathogens in the causation of chronic enteritis of the Crohn's disease type in humans, as do recent results from CD research in Europe and North America (Schwartz *et al.* 2000; Bull *et al.* 2003; Naser *et al.* 2004; Romero *et al.* 2005).

The nucleotide polymorphisms we found at positions 44, 45 and 125 in the AV1/AV2 amplicons from *M. avium* subspecies *paratuberculosis* in Chinese patients correspond to nucleotides 120, 121 and 201 of IS*900*. They differ from the polymorphisms reported in *M. avium* subspecies *paratuberculosis* from CD patients in the USA (Naser *et al.* 2004) as well as from IS*900* sequences in GenBank. This is in agreement with differences in *M. avium* subspecies *paratuberculosis* isolates from animals already identified between Australia and Europe and Argentina and Europe (Moreira *et al.* 1999; Whittington *et al.* 2001) and is consistent with diversification also of human strains between continents. Larger studies of *M. avium* subspecies *paratuberculosis* infection in CD patients in China using fresh tissues and optimized PCR and culture methods together with genotyping (Amonsin *et al.* 2004; O'Shea *et al.* 2004; Ghadiali *et al.* 2004) are needed.

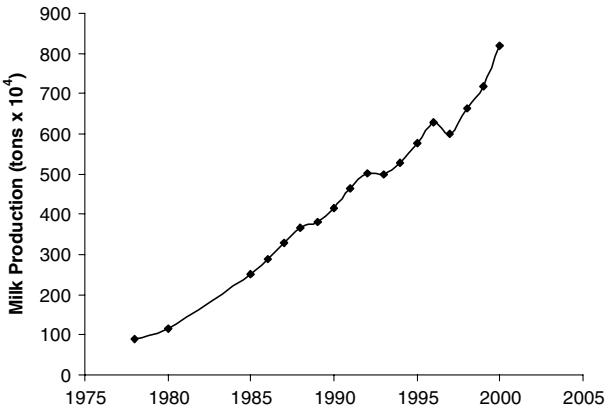

Figure 2. Milk production in China from 1978 to 2000.

The present preliminary study found that a minority proportion of people in China without inflammatory bowel disease are colonized by *M. avium* subspecies *paratuberculosis*. Together with *M. avium* subspecies *paratuberculosis* infecting Chinese people with CD, this shows that people in China are exposed to these pathogens. In Europe, North America and Argentina where infection in domestic livestock is widespread and well documented (Manning & Collins 2001) *M. avium* subspecies *paratuberculosis* is transmitted to human populations in retail pasteurized milk (Millar *et al.* 1996; Grant *et al.* 2002; Wuhib *et al.* 2004; http://www.johnes.org). *M. avium* subspecies *paratuberculosis* can survive for long periods in the environment (Larsen *et al.* 1956; Riemann & Abbas 1983; Whittington *et al.* 2004; Pickup *et al.* 2005). In some areas people are also at risk from contaminated surface waters (Pickup *et al.* 2005). There are few published studies on Johne's disease in China but the information available would suggest that *M. avium* subspecies *paratuberculosis* infection in domestic livestock does occur and may have been increased by the importation of infected animals (Ke *et al.* 1998). Figure 2 shows that the annual production of cow's milk in China has increased by almost 10-fold over the period 1978–2000. Milk consumption especially in school children has also increased (Du *et al.* 2004; Fraser 2004; Liu *et al.* 2004; Zhang *et al.* 2004). Crohn's disease particularly in young people is increasing in China (Sung *et al.* 1994; Leong *et al.* 2004; Zheng *et al.* 2004). Our data suggest that there is a substantial public health need to determine whether viable *M. avium* subspecies *paratuberculosis* is present in retail milk in China and to assess the burden of subclinical as well as clinical infection in farm animals.

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