Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*

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SUMMARY

We have used nuclear (ribosomal ITS1) and mitochondrial (ND1) sequences to characterize human and pig isolates of *Echinococcus granulosus* collected by fine needle aspiration biopsy (FNAB) in Poland. The data indicate clearly that the Polish patients were not infected with the common sheep strain (G1 genotype) of *E. granulosus*, normally associated with human cystic hydatid infection. Instead, the hydatid parasite infecting the Polish patients shares very similar ND1 sequence with the previously characterized pig (G7) genotype but it also exhibits some clear differences. In particular, *E. granulosus* DNA from the Polish patients amplified a single ITS1 fragment in PCR and distinct ITS1–RFLP patterns were obtained after restriction digestion. The form of hydatid isolated from the Polish patients appears, therefore, to represent a distinct, previously undescribed genotype (designated G9) of *E. granulosus*.

Key words: *Echinococcus granulosus*, human hydatid disease, cystic hydatidosis, NADH dehydrogenase I gene, PCR-RFLP analysis.

INTRODUCTION

There is considerable information about genetic variation within the recognized species of *Echinococcus*, with intraspecific variation being particularly well studied in *E. granulosus*; a number of distinct strains are now recognized (Bowles & McManus, 1993*a*; Thompson, 1995). Recently, analysis of 2 mitochondrial genome marker genes, cytochrome *c* oxidase 1 (COI) (Bowles, Blair & McManus, 1992*a*) and NADH dehydrogenase 1 (NDI) (Bowles & McManus, 1993*b*) detected 7 distinct genotypes (referred to as G1–G7) among *E. granulosus* with the genotypically defined groups being in accord with biologically and morphologically defined *E. granulosus* strains. Subsequently, data from the CO1 and ND1 gene regions became available for 4 isolates (designated G8) of the cervid strain or 'northern form' of *E. granulosus* (Bowles, Blair & McManus 1994). Furthermore, a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method has been developed which allows *E. granulosus* isolates to be distinguished easily and rapidly using size and sequence of the nuclear rDNA ITS1 region as a genetic marker (Bowles & McManus, 1993*c*).

Previous study of Polish hydatid material has concentrated on characterizing *E. granulosus* isolated from pigs. This work has suggested that the form occurring in Polish and other European pigs may represent a distinct strain that can be separated morphologically and genetically from other strains, and which exhibits features of epidemiological significance, including a rapid rate of development in dogs and an apparent low infectivity to humans and domestic ungulates (Eckert *et al.* 1993). Conventional RFLP analysis had earlier revealed distinct hybridization profiles with DNA from isolates of *E. granulosus* from pigs in Poland and the former Yugoslavia (McManus & Rishi, 1989). Furthermore, the accrued mitochondrial DNA sequence data has now allowed the grouping of pig isolates into a distinct genotype (G7) although sequences for the camel strain (G6) are very similar (Bowles *et al.* 1992*a*; Bowles & McManus, 1993*b*).

Here we have used several molecular genetic methods to characterize isolates of *E. granulosus* from Poland, with the principal aim being to genotype human isolates in an area where sheep are rarely bred and where the pig strain of *E. granulosus* is the most common form found in domesticated animals (Pawlowski, 1985; Lis, 1988). The human material was collected by fine needle aspiration biopsy (FNAB), a new procedure which, along with ultrasonography, allows differential diagnosis of suspected hepatic cysts in the liver, and permits collection of parasite material for analysis from

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Table 1. Clinical and other features of the 10 human *Echinococcus granulosus* isolates (all from Poland; all with simple cysts in the liver) analysed in the study

(F, female; M, male; P, Poznan; O, outside Poznan; Fw, farmworker; Ww, white collar worker; R, rural; U, urban.)

Patient	G.S.	K.E.	O.K.	C.E.	M.R.	N.M.	N.T.	A.E.	B.R.	K.A.
Age (years)	65	57	26	40	35	39	43	45	27	58
Sex	F	F	F	F	F	F	F	F	M	M
Province	P	P	O	P	O	P	P	O	P	P
Profession	Fw	Ww	Ww	Ww	Ww	Ww	Fw	Fw	Fw	Fw
Area lived	R	U	R	R	U	U	R	R	R	R
Symptoms	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cyst size (cm)	5	7·5	5·7	4	4·8	5·4	4·6	3·9	8·5	6·5
Hooks in biopsy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Gharbi class*	IV	II	IV	IV	IV	I	I	IV	IV	II
Positive serology†	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No	No
DH test‡	+	+	+	+	+	+	+	+	+	+

* Internationally accepted classification (groups I–V) based on ultrasonographic examination of hydatid cysts in the liver (see Gharbi *et al.* 1981).
† By ELISA and/or indirect haemagglutination assay, and measurement of specific IgG4/IgE.
‡ Delayed hypersensitivity test; +, hyporeactivity (see Kacprzak & Stefaniak, 1995).

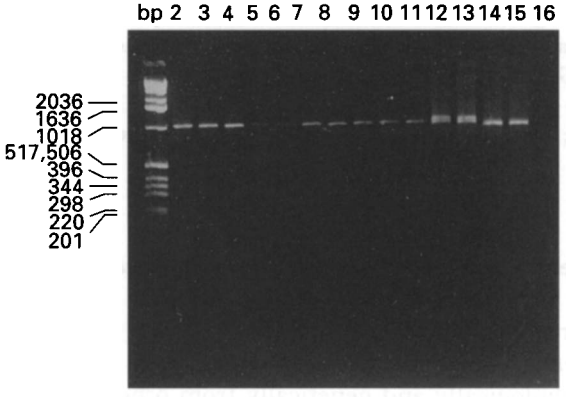

Fig. 1. Comparison of PCR-amplified ITS1 fragments from Polish human isolates with the pig (G7 genotype, 2 isolates) and common sheep (G1 genotype, 2 isolates) strains of *Echinococcus granulosus*. Note the G1 (0·9 and 1 kb) and the G7 genotypes (1 kb and 1·1 kb) have 2 fragments of different sizes (see also Bowles & McManus, 1993*c*) whereas the Polish human isolates all produced a single fragment of 1·04 kb. Lane 1, molecular weight markers (bp); lanes 2–11, Polish human isolates; lanes 12, 13, pig strain (G7 genotype, different isolates); lanes 14, 15, sheep strain (G1 genotype, 2 different isolates); lane 16, control lane, no template DNA.

patients residing in areas where hydatid disease is relatively uncommon (Stefaniak & Lemke, 1995).

MATERIALS AND METHODS

E. granulosus *material*

Genetic analysis was performed on protoscoleces/brood capsules isolated from 10 hydatid patients (10 isolates; clinical and other details are shown in Table 1), hospitalized in the Clinic of Parasitic and Tropical Diseases, Poznan, Poland, and 7 pigs (8 isolates, numbered 7, 9, 15, 19, 44, 48, 53 and 58) slaughtered in a Poznan abattoir. The pigs were Polish White race, 8 months old, weighing between 90 and 105 kg. Isolates 9, 19 and 48 originated from 3 hydatid cysts (all > 4 cm in diameter) infecting 3 pigs raised at 3 different commercial pig farms; isolates 53 and 58 originated from 2 similar sized cysts infecting a single pig raised on a non-commercial farm. Due to the quality of the starting material, only limited genetic information was obtained from the 3 additional pig isolates (7, 15, 44) collected from commercial pig farms. The human isolates were taken from hydatid cysts by fine needle aspiration biopsy (FNAB), performed under US guidance, by the transhepatic route and under albendazole cover (Stefaniak & Lemke, 1995). The pig isolates were also collected after slaughter from infected livers by FNAB. The human and pig isolates were preserved in 70% ethanol (McManus & Rishi, 1989) and transported to the Brisbane laboratory for analysis.

Total genomic DNA was extracted from the alcohol-preserved *E. granulosus* isolates using standard extraction procedures (Maniatis, Fritsch & Sambrook, 1989). Purified DNA samples, isolated in identical fashion, from the common *E. granulosus* sheep (G1 genotype) and pig (Poland; G7 genotype) strains (see Bowles *et al.* 1992*a*) were available for comparative purposes. We were unable to carry out complete genetic analysis on some of the isolates because of the relatively small quantity and/or the quality of parasite material which resulted in limited amounts of extractable DNA for study.

PCR–RFLP analysis of nuclear rDNA ITS1 region

E. granulosus genomic DNA samples, including those of the standard G1 and G7 genotypes, were

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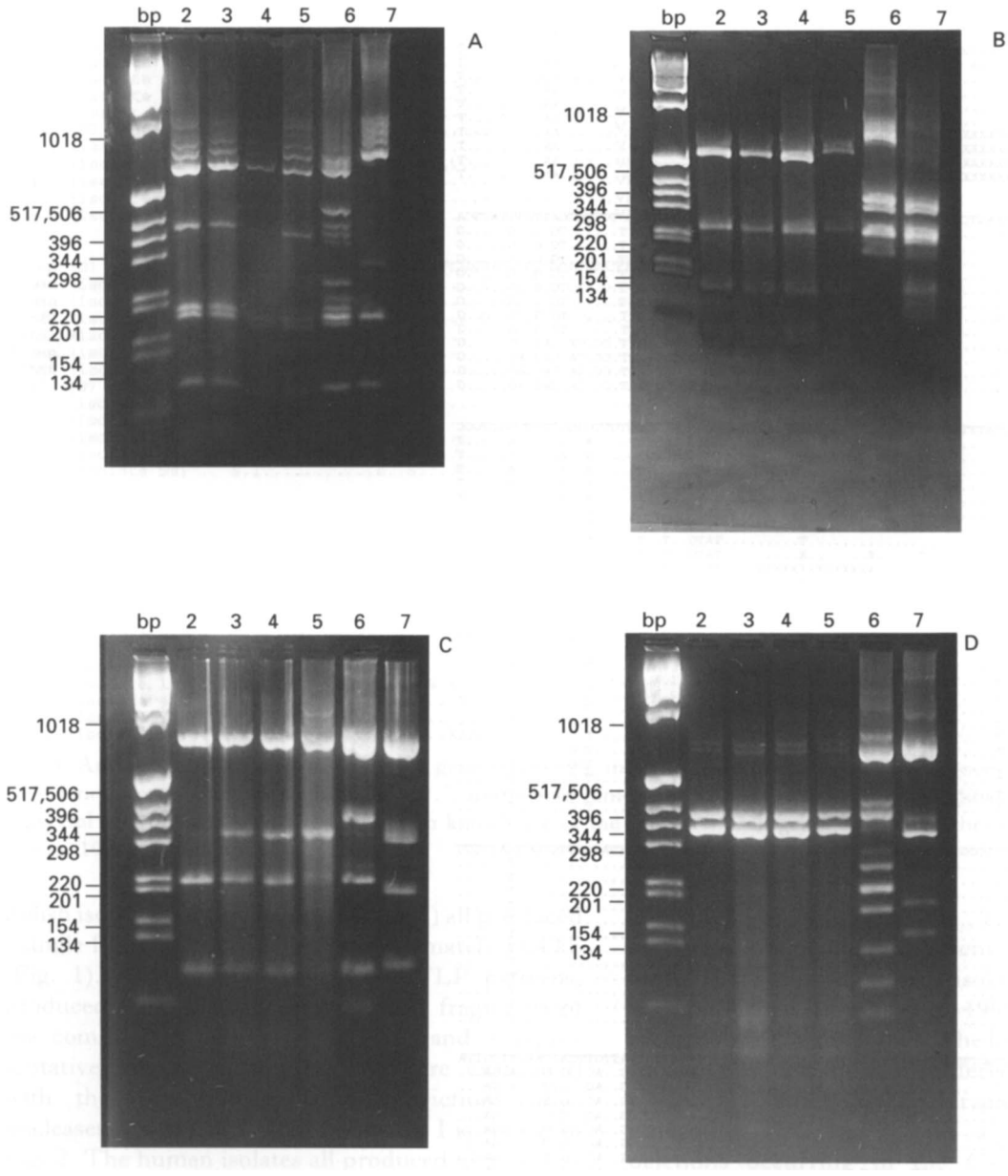

Fig. 2. Comparison of ITS1–PCR–RFLP patterns for *Echinococcus granulosus* G1 (sheep) genotype, G7 (pig) genotype and representative (G.S., O.K., K.E., N.T.) Polish human isolates. ITS1–PCR products were digested with (A) *Alu* I, (B) *Cfo* I, (C) *Msp* I or (D) *Rsa* I. Lane 1, molecular weight markers (bp); lanes 2–5, 4 human Polish isolates; lane 6, G7 genotype; lane 7, G1 genotype. Although the patterns were not absolutely invariant within the human isolates with some of the restriction enzymes, they were, nevertheless, clearly quite distinct from the G1 and G7 profiles. With *Alu* I (A), the multiple fragments above approximately 650 bp are probably due to overdigestion or non-specific digestion of the ITS1–PCR products.

analysed using a direct PCR-based approach described by Bowles & McManus (1993*c*).

Mitochondrial ND1 gene sequencing

Sequences within the mitochondrial NDI gene were obtained for the *E. granulosus* genomic DNA samples, including DNA samples of the standard G1 and G7 genotypes, using the Applied Biosystems Taq DyeDeoxy™ Terminator Cycle sequencing kit and Perkin Elmer Cetus DNA Thermal Cycler 480. Sequence was obtained from duplicate or triplicate samples where sufficient DNA was available for analysis.

RESULTS

PCR–RFLP analysis of the nuclear rDNA ITS1 region

Two ITS1 fragments of different sizes are routinely amplified from DNA of any isolate of the *E. granulosus* sheep (G1 genotype) (1 kb and 0·9 kb) and pig (G7 genotype) (1 kb and 1·1 kb) strains (see Bowles & McManus, 1993*c*). In contrast, the human

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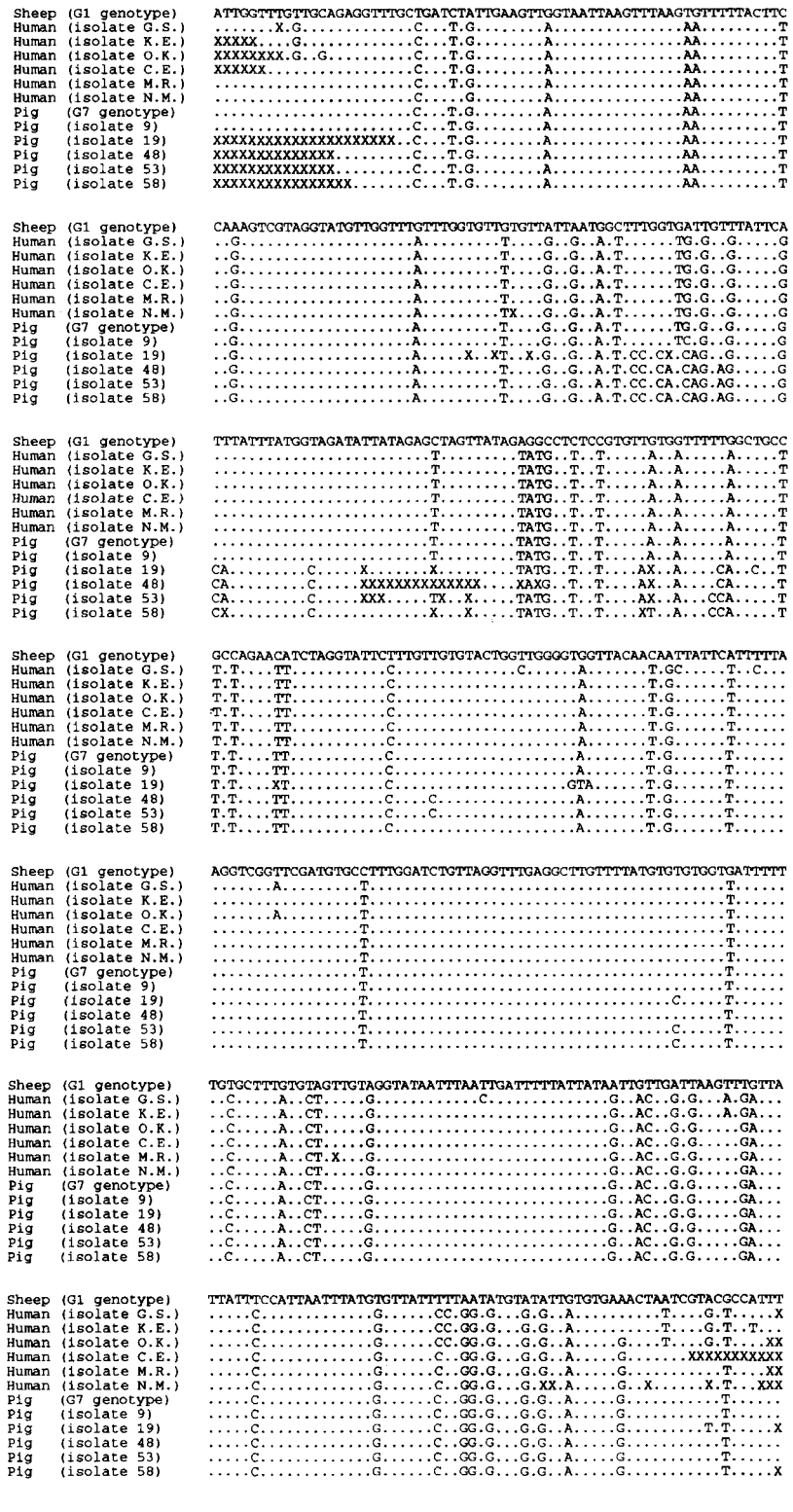

Fig. 3. Nucleotide sequences of a fragment (471 bp) of the mitochondrial ND1 gene compared for the common sheep (G1) genotype, pig (G7) genotype, 6 Polish human isolates and 5 recently collected Polish pig isolates of *Echinococcus granulosus*. 'X', sequence not obtained. '.', nucleotide same as top line (G1 genotype). Restricted sequence (due to

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Sheep (G1 genotype)   IGLLQSFADLLKLVIKFKCFYFQSRSYVGLFGVVLLMALVIVYSFIYGSYYSASYSGLSVLWFLAA
Human (isolate G.S.)  ..XV.............N.............L...V..V..........V...M.........
Human (isolate K.E.)  XX.V.............N.............L...V..V..........V...M.........
Human (isolate O.K.)  XXXVE............N.............L...V..V..........V...M.........
Human (isolate C.E.)  XX...............N.............L...V..V..........V...M.........
Human (isolate M.R.)  ...............N.............L...V..V..........V...M.........
Human (isolate N.M.)  ...............N.............X...V..V..........V...M.........
Pig   (G7 genotype)   ...............N.............L...V..V..........V...M.........
Pig   (isolate 9)     ...............N.............L...V..L..........V...M.........
Pig   (isolate 19)    XXXXXXX.........N.............LX..VPXQ...H....X..X...M...X..S..
Pig   (isolate 48)    XXXXX...........N.............L...VPQQE..H....XXXXXXXM...X..S..
Pig   (isolate 53)    XXXXX...........N.............L...VPQQE..H....XX..X.SM...X..P..
Pig   (isolate 58)    XXXXXX..........N.............L...VPQQE..H.......XX.SM...X..P..

Sheep (G1 genotype)   ASTSRYSLLCTGWGGYNNYSFLSSVRCAFGSVSFEACFMCVVIFCALCSCSYNLIDFYYNCWLSLL
Human (isolate G.S.)  S.I..........S..S.....D.....................C.G...T....SY.W.W.
Human (isolate K.E.)  S.I..........S..S.............................C.G........SY.W.W.
Human (isolate O.K.)  S.I..........S..S.....D.....................C.G........SY.W.W.
Human (isolate C.E.)  S.I..........S..S.............................C.G........SY.W.W.
Human (isolate M.R.)  S.I..........S..S.............................X.G........SY.W.W.
Human (isolate N.M.)  S.I..........S..S.............................C.G........SY.W.W.
Pig   (G7 genotype)   S.I..........S..S.............................C.G........SY.W.W.
Pig   (isolate 9)     S.I..........S..S.............................C.G........SY.W.W.
Pig   (isolate 19)    S.X..........Y..S.............................C.G........SY.W.W.
Pig   (isolate 48)    S.I.....S.....S..S.............................C.G........SY.W.W.
Pig   (isolate 53)    S.I.....S.....S..S.............................C.G........SY.W.W.
Pig   (isolate 58)    S.I..........S..S.............................C.G........SY.W.W.

Sheep (G1 genotype)   LFPLIYVLFLMCMLCETNRTPFDYG
Human (isolate G.S.)  ......G...V.V....I.A..XXX
Human (isolate K.E.)  ......G...V.V....I.A..YXX
Human (isolate O.K.)  ......G...V.V....I.A.XXXX
Human (isolate C.E.)  ......G...V.V.....XXXXXXX
Human (isolate M.R.)  ......G...V.V........XXXX
Human (isolate N.M.)  ......G...V.VX.....X.XXXX
Pig   (G7 genotype)   ......G...V.V............
Pig   (isolate 9)     ......G...V.V............
Pig   (isolate 19)    ......G...V.V......S.X..X
Pig   (isolate 48)    ......G...V.V.........X..
Pig   (isolate 53)    ......G...V.V...........X
Pig   (isolate 58)    ......G...V.V........XXXX
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Fig. 4. Amino acid sequences of the ND1 gene fragments, inferred from the nucleotide sequences presented in Fig. 3. 'X', denotes an unidentified amino acid; '.', amino acid same as top line (G1 genotype). Modifications of the universal genetic code were used, based on knowledge of the mitochondrial genetic code in other organisms (see Caron, 1990).

Polish isolates of *E. granulosus* (Table 1) all produced a single ITS1 PCR product of approximately 1·04 kb (Fig. 1). A comparison of PCR–RFLP patterns, produced after digestion of the ITS1 fragments of the common sheep (G1), pig (G7) and 4 representative human isolates (all 10 were examined) with the 4-base recognizing restriction endonucleases *Alu* I, *Cfo* I, *Msp* I and *Rsa* I is shown in Fig. 2. The human isolates all produced identical or very similar patterns with the individual enzymes but the patterns were substantially different to those obtained with the G1 and G7 DNA samples. The *E. granulosus* ITS1 region of these genotypes does not have a *Taq* 1 restriction site and hence the PCR products were not digested by this enzyme (data not shown). The Polish human isolates were clearly distinguishable from the sheep (G1) and camel(G6)/pig(G7) strains and all other *Echinococcus* genotypes (see Bowles & McManus, 1993*c*; Bowles *et al.* 1994) examined (data not shown).

In preliminary studies (data not shown, but sequence has been deposited in GenBank), ITS1 sequence (technical details described by Bowles, Blair & McManus, 1995) was obtained for 3 (G.S., approx. 1 kb; K.E. and O.K., each approx. 0·5 kb, sequence obtained for 2 different regions of the same area) of the Polish human isolates and compared with published (Bowles *et al.* 1995) ITS1 sequences for *Echinococcus* genotypes. The human *E. granulosus* sequence is considerably different from the sheep strain (G1) small (0·9 kb) fragment (14% actual nucleotide differences with a number of large deletions occurring in the G1 sequence, which reflects its smaller size (0·9 kbp, compared with 1·04 kb) and is most similar to the camel strain (G6) small (1·0 kb) fragment (4% nucleotide differences).

Mitochondrial ND1 gene sequence

Partial nucleotide sequences and predicted amino acid sequences were obtained for the ND1 genes of 6 human Polish isolates of *E. granulosus* and 5 isolates recently collected from pigs from Poland and aligned with published (Bowles & McManus, 1993*b*; Bowles *et al.* 1994) ND1 sequences for *Echinococcus* genotypes. A comparison of aligned nucleotide (Fig. 3) and predicted amino acid sequences (Fig. 4) is shown for the Polish human and pig isolates and is

lack of DNA), approximately 100–150 bp at the 5′ end of the gene, was also obtained for an additional 3 pig (isolates 7, 15 and 44) and 3 human isolates (K.A., B.R., A.E.) but these sequences have not been included in the alignment. The available, additional human isolate sequences were identical to those shown in the alignment while those of the additional pig *E. granulosus* sequences were virtually identical to those of isolates 19, 48, 53 and 58.

compared with sequence obtained for the common sheep (G1 genotype) and pig (G7 genotype) strains. The newly obtained G1 and G7 ND1 sequences are identical to those earlier published (Bowles & McManus, 1993*b*). Minor nucleotide differences, with some resultant amino acid changes, were evident between some of the human isolate sequences which were otherwise highly conserved. The human isolate sequences obtained are very different from the sheep strain (G1) (15% nucleotide differences; 15–16% amino acid differences) but are virtually identical to the pig strain (G7). Apart from a single (C/T) nucleotide difference, 1 of the pig isolates (isolate 9) had identical sequence to the G7 genotype sequence, obtained for 2 Polish pig isolates collected earlier (Bowles & McManus 1993*b*). The others (isolates 19,48,53,58) had very similar sequences to the G7 genotype although some minor variation was evident, particularly in one region at the 5′ end of the ND1 gene which results in 7 (= 4%) amino acid differences (Figs 3 and 4).

DISCUSSION

The nuclear and mitochondrial gene markers used in the current study have previously been used successfully in molecular epidemiological surveys on *E. granulosus* to confirm the presence and reveal the host preferences of sheep (G1 genotype) and camel (G6 genotype) strains in Kenya (Wachira *et al.* 1993), to show that a single strain (G1 genotype) of *E. granulosus* cycles in domestic and sylvatic hosts on mainland Australia (Hope *et al.* 1991, 1992), and to prove that the cattle strain (G5 genotype) is infective to humans (Bowles, Van Knapen & McManus, 1992*b*). The findings of each of these studies have considerable implications for public health.

A direct PCR-based RFLP approach targetting the internal transcribed spacer 1 (ITS1) of the rDNA repeat provides a rapid method for discriminating *Echinococcus* and strains (Bowles & McManus, 1993*c*). Using this procedure, *E. granulosus* DNA of the common sheep (G1) and pig (G7) genotypes produce 2 different (large and small) ITS1 PCR products (Bowles & McManus, 1993*c*) but, in contrast, the human Polish isolates analysed here yielded a single ITS1–PCR fragment of approximately 1·04 kb. Subsequent PCR–RFLP patterns produced after digestion of the ITS1–PCR products showed that *E. granulosus* from the Polish patients was readily distinguishable from the common sheep (G1), and camel(G6)/pig(G7) genotypes and all other *Echinococcus* genotypes previously examined (see Bowles & McManus, 1993*c*; Bowles *et al.* 1994).

In preliminary studies, DNA sequence, obtained for the ITS1 region of 3 of the Polish human *E. granulosus* isolates, was shown to be very different from that published earlier for the sheep strain (G1) small (0·9 kb) fragment (Bowles *et al.* 1995). In like fashion, the human Polish *E. granulosus* ND1 sequence is very different from that of the sheep strain (G1), being most similar to the pig strain (G7).

Collection of human cystic material is generally problematical, except in hospitals located in highly endemic *E. granulosus* areas such as North Western China, where surgery for removal of hydatid cysts is commonly performed. However, in these areas, the sheep strain (G1 genotype) prevails (McManus, Ding & Bowles, 1994). In other regions where human cystic hydatid disease is uncommon, FNAB is the only method available, for collecting for analysis, a sufficient number of isolates from patients with space-occupying lesions in the liver, suspected as being due to *E. granulosus*. The results reported here indicate clearly that the Polish patients from whom hydatid material was collected by FNAB, were not infected with the common sheep strain of *E. granulosus* normally associated with human infection (McManus & Smyth, 1986). There is only 1 previously reported case – an 11-year-old Dutch boy who harboured the genetically distinct cattle (G5) form – of a patient being shown infected, by molecular analysis, with a hydatid parasite other than the sheep strain (Bowles *et al.* 1992*b*).

It had been suspected, on circumstantial grounds, that *E. granulosus* from pigs has low infectivity to humans (Pawlowski, 1985; Pawlowski *et al.* 1993; Eckert *et al.* 1993) but this needed to be confirmed by identification of isolates taken from humans residing in areas where pig hydatidosis is highly prevalent. The hydatid parasite infecting the Polish patients shares molecular affinity with the previously characterized pig (G7) genotype but it exhibits some clear differences – in particular, a single ITS1 fragment amplified by PCR and unique RFLP patterns obtained after restriction digestion of the fragment – and appears to represent a distinct genotypic group, which we designate G9, of *E. granulosus*.

The major question now arising concerns the reservoir(s) of human hydatid disease in Poland. It is unlikely to be sheep in Poznan Province as ovine infections with *E. granulosus* are rarely seen there, whereas the prevalence of echinococcosis in pigs is higher than in other parts of the country (Pawlowski *et al.* 1993). In Poland in 1985, the national figures for cystic hydatidosis in slaughtered animals showed prevalences of 5·35% in pigs, 1·08% in sheep and 0·04% in cattle (Lis, 1988). Earlier genetic analysis has shown that sheep harbour only the G1 genotype or closely related G2 genotype (in Tasmania). It is more likely that the pig naturally harbours the newly identified G9 genotypic group described here although, unlike in humans, these parasites may develop poorly, producing small, yet viable cysts, in this host. The hydatid material we analysed of porcine origin originated from large, well-developed cysts which, although exhibiting some minor ND1

heterogeneity, could be readily categorized into the G7 (pig strain) genotypic grouping we have previously defined; it may thus be that much smaller pig cysts, representing the G9 genotype, have yet to be processed and typed.

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