Molecular Identification of *Echinococcus granulosus* in Tunisia: First Record of the Buffalo Strain (G3) in Human and Bovine in the Country

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Abstract: It has been demonstrated that human hydatidosis was generally due to G1 genotype of *Echinococcus granulosus* throughout the world. Nevertheless, some other genotypes, such as G3, were recently identified for human cyst. The present work confirms the predominance of the sheep strain G1 in humans, bovine and ovine and demonstrates for the first time the occurrence of the buffalo strain G3 in human and bovine in Tunisia.

Keywords: *Echinococcus granulosus*, Tunisia, hydatidosis, echinococcosis, CO1, buffalo strain, human infection, bovine, G3 genotype, North Africa.

INTRODUCTION

Cystic echinococcosis (CE) or hydatidosis is a widespread zoonose in the world with significant socio-economic repercussions. In Tunisia, CE represents a considerable public health problem with an annual surgical incidence of 15/100 000 inhabitants [1]. Hydatidosis is of veterinary and medical importance because infection with metacestode may cause severe illness and high economic losses. Thus, in Tunisia the annual cost of this disease in both human and animals is approximately US$ 15 million [2]. Although exceptions occur in East Africa [3], humans are usually a dead-end host and the disease is preferentially located in the liver and the lung.

The definitive host is almost always a canid and a many intraspecific variants or strains of *E. granulosus* have been described from different intermediate host species as genotype G1 to G10 [4-8]. More recently, a molecular phylogeny of Echinococcus spp. was established from the complete mitochondrial genomes [9], and *E. granulosus* sensu lato was split into *E. granulosus* sensu stricto (genotypes G1–G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5) and *E. canadensis* (genotypes G6–G10).

Our previous study using restriction fragment length polymorphism [10], demonstrated that there are only two genotypes in Tunisia: the common sheep strain G1 which is predominant and infects humans as well as bovine and ovine and the G6 genotype restricted to the camel.

The present work confirms the predominance of the sheep strain G1 in humans, bovine and ovine and demonstrates for the first time the occurrence of the buffalo strain G3 in human and bovine in Tunisia.

MATERIAL AND METHODS

Parasite Material

In this work, we have analyzed 20 fertile cysts (10 ovine and 10 bovine) collected from the abattoir of Sousse (Middle of Tunisia). Ten fertile cysts from children aged 4–10 years old operated at Monastir Teaching Hospital were also analysed.

Protoscoleces were removed from metacestode by several washings with a sterile solution of sodium chloride 0.9% followed by sedimentation at room temperature [10]. Then, each sediment of protoscoleces was homogenized with an equal volume of distilled water and freezed overnight in liquid nitrogen. Total DNA of samples were extracted using phenol/chloroform protocol [11].

Mitochondrial Cytochrome c Oxydase (CO1) Gene Sequencing

CO1 was amplified by PCR from each individual sample of *E. granulosus* DNA. PCR reaction was performed as described by Gasser *et al.* [12]. The verified double stranded CO1 sequences were obtained from Genome Express (Meylan, France) using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Consensuses were built

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with SeqMan software (DNAStar, Inc. Madison, WI). Multiple sequence alignments were made with ClustalW method using MegAlign software (DNAStar) and compared with previously published sequences of *E. granulosus* [U50464 (G1), M84662 (G2), M84663 (G3), M84664 (G4), M84665 (G5), M84666 (G6), M84667 (G7), and AF525457 (G10)].

RESULTS

The sequencing of the mitochondrial CO1 gene of 30 *E. granulosus* isolates produced unambiguous sequences of 366 bp for each sample analyzed. For one human sample (KH59) originating from a rural area of Kairouan (Middle of Tunisia) and one bovine isolate (PB30), alignment of the obtained sequences with the G1 to G8 and the G10 genotypes showed that hydatid cysts were produced by G3 buffalo strain (Fig. **1**). If the human cyst showed a total homology with the G3 sequence (GenBank **M84663**), the bovine isolate sequence presented 99% identity to this genotype. The difference was a transversion mutation of a cytosine to a thymine in position 44 (C44T) resulting in a substitution of the alanine in position 15 by a valine according to the echinoderm mitochondria genetic code [13]. The PB30 CO1 gene sequence was registered in GenBank (**AY850565**). Furthermore, for 19 isolates (8 sheep, 8 humans and 3 cattle), sequences obtained presented a total homology with the common sheep strain G1 (GenBank **U50464**). For the 9 remaining isolates (2 sheep, 1 human and 6 cattle), sequences obtained presented 99% homology with the G1 genotype (sheep strain). The difference was a transversion mutation of a cytosine to a thymine in position 56 (C56T) resulting in a substitution of the alanine in position 27 by a valine according to the echinoderm mitochondria genetic code [13]. We noted that all cysts analyzed presented a high fertility with viable protoscoleces.

DISCUSSION

Cystic echinococcosis constitutes a serious public health problem in Tunisia despite the deployed prevention programs. The characterisation of the strains responsible for

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                  10                  20                  30                  40                  50
      ------------------+-------------------+-------------------+-------------------+-------------------+-
    1 C C T G G A T T T G G T A T A A T T A G T C A T A T T T G T T T G A G T A T T A G T G C T A A T T T  COI G3
    1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . .  PB30
    1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . .  KH59

                  60                  70                  80                  90                  100
      ------------------+-------------------+-------------------+-------------------+-------------------+-
   57 T G A T G C G T T T G G G T T T T A T G G G T T G T T G T T T G C T A T G T T T T C T A T A G T G T  COI G3
   57 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
   57 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  110                 120                 130                 140                 150
      ------------------+-------------------+-------------------+-------------------+-------------------+-
  101 G T T T G G G T A G C A G G G T T T G G G G T C A T C A T A T G T T T A C T G T T G G G T T G G A T  COI G3
  101 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
  101 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  160                 170                 180                 190                 200
      ------------------+-------------------+-------------------+-------------------+-------------------+-
  151 G T G A A G A C G G C T G T T T T T T T T A G C T C T G T T A C T A T G A T T A T A G G G G T T C C  COI G3
  151 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
  151 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  210                 220                 230                 240                 250
      ------------------+-------------------+-------------------+-------------------+-------------------+-
  201 T A C T G G T A T A A A G G T G T T T A C T T G G T T A T A T A T G T T G T T G A A T T C G A G T G  COI G3
  201 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
  201 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  260                 270                 280                 290                 300
      ------------------+-------------------+-------------------+-------------------+-------------------+-
  251 T T A A T G C T A G T G A T C C G G T T T T G T G A T G G G T T G T T T C T T T T A T A G T G T T G  COI G3
  251 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
  251 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  310                 320                 330                 340                 350
      ------------------+-------------------+-------------------+-------------------+-------------------+-
  301 T T T A C G T T T G G G G G A G T T A C G G G T A T A G T T T T G T C T G C T T G T G T G T T A G A  COI G3
  301 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  PB30
  301 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  KH59

                  360
      ------------------+-------------
  351 T A A T A T T T T G C A T G A T  COI G3
  351 . . . . . . . . . . . . . . . .  PB30
  351 . . . . . . . . . . . . . . . .  KH59
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Fig. (1). Alignment Report of COI genotype G3 (GB# **M84663**), PB30 (bovine cyst, GB# **AY850565**) and KH59 (human cyst). *Legend*: Nucleotide sequences of a fragment (366 bp) of the *Echinococcus granulosus* mitochondrial cytochrome c oxydase I gene (COI) for the G3 genotype, PB30 and KH59 were aligned by ClusalW method (MegAlign software, DNAStar®). Dots indicate nucleotides that are conserved between the G3 genotype, PB30 and KH59 sequences.

human echinococcosis in Tunisia is nowadays a significant point which has to be taken into consideration in order to focus and to adapt the control measures and the means of diagnosis. Indeed, it was shown that virulence and infectivity to humans as well as the antigenicity were variable depending on the strain of *E. granulosus* involved [14].

With 28 isolates out of 30 typed as G1 genotype, the result of the current study are in agreement with those of our previous study attributing all human, sheep and bovine cysts to the ovine strain G1 [10].

Nevertheless, the results obtained here reveal for the first time the presence of *E. granulosus* buffalo strain (G3) in Tunisia. While buffalo strain was previously detected in buffalo, bovine and sheep in Italy [15] and Turkey [16] our results represent the first report of the presence of this strain in Tunisia.

Furthermore, this study represents the second confirmed case of human infection by the G3 genotype, the first case was previously reported in Italy [15]. The fertility of the cysts analyzed and the viability of extracted protoscoleces demonstrate the perfect adaptation of this strain to man. Contamination by this strain is apparently sporadic but it's certainly not an isolated case. Indeed, this patient comes from a rural area of Kairouan (Centre of Tunisia) where there are frequent contacts between animals, dogs and humans. Moreover, this region is an hyperendemic area (annual surgical incidence of 41.5/100 000 inhabitants [1, 17] characterised by a very high canine contamination (45.7%) [18]. Thus, it would be surprising that only one child is contaminated by this strain which seems to be well adapted to humans

Regarding bovine, the molecular analysis of cyst PB30 revealed a C44T mutation when compared to the G3 genotype. This type of mutation represents either a genotypic variant or a distinct strain which resembles the G3 genotype. However, no data concerning the infectivity, the virulence or the antigenicity of this isolate may allow us to give this mutation some particular phenotypic characteristics. Consequently, we consider this mutation as a genotypic variant of the G3 genotype and it belongs to *E. granulosus* sensu stricto [9].

The fact that the bovine is infested by a genotypic variant of this strain allows us to assume that this animal could be the intermediate host which maintains this particular strain in Tunisia. Indeed, contrary to what was reported in India [4, 19] and in Italy [20], the intermediate host cannot be the buffalo since there is no buffalo breeding in Tunisia. Nevertheless, the isolation of this genotype remains possible in other Tunisian intermediate hosts as it has already been described in ovine and bovine cyst in Italy [15] and Turkey [16].

In the current state of knowledge, it is impossible to determine the origin and importance of this strain in Tunisia. Due to the complexity of Tunisian trade-circuits, no information concerning the origin and travelling zones of the slaughtered intermediate hosts is available. Furthermore, only one human isolate was typed as a G3 genotype and consequently we cannot conclude on the role of this genotype in human hydatid disease in Tunisia and further study is necessary in order to evaluate the real risk that it represents for the public health.

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