Current status of the genetics and molecular taxonomy of *Echinococcus* species

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SUMMARY

The taxonomy of *Echinococcus* has long been controversial. Based mainly on differences in morphology and host-parasite specificity characteristics, 16 species and 13 subspecies were originally described. Subsequently, most of these taxa were regarded as synonyms for *Echinococcus granulosus* and only 4 valid species were recognised: *E. granulosus*; *E. multilocularis*; *E. oligarthrus* and *E. vogeli*. But, over the past 50 years, laboratory and field observations have revealed considerable phenotypic variability between isolates of *Echinococcus*, particularly those of *E. granulosus*, which include differences in: morphology in both larval and adult stages, development *in vitro* and *in vivo*, host infectivity and specificity, chemical composition, metabolism, proteins and enzymes, pathogenicity and antigenicity. The application of molecular tools has revealed differences in nucleic acid sequences that reflect this phenotypic variation and the genetic and phenotypic characteristics complement the previous observations made by the descriptive parasitologists many years ago. The fact that some of these variants or strains are poorly or not infective to humans has resulted in a reappraisal of the public health significance of *Echinococcus* in areas where such variants occur. A revised taxonomy for species in the *Echinococcus* genus has been proposed that is generally accepted, and is based on the new molecular data and the biological and epidemiological characteristics of host-adapted species and strains.

Key words: *Echinococcus*, strain, genetics, molecular taxonomy, mitochondrial genome, phylogeny, taxonomic revision.

INTRODUCTION

The classification of *Echinococcus* has long been controversial because of a lack of phenotypic characters, inadequate taxonomic descriptors and lack of evidence for geographical or ecological segregation (reviewed in Thompson *et al*. 1995; Thompson, 2001; Thompson and McManus, 2002). Based mainly on host-parasite specificity characteristics, many species and subspecies of *Echinococcus* were described originally (Ortlepp, 1934; Lopez-Neyra and Soler Planas, 1943; Williams and Sweatman, 1963; Verster, 1965). However, most of these taxa were regarded as synonyms for *Echinococcus granulosus* (Rausch, 1967), and subsequent taxonomic revisions recognised only four valid species: *E. granulosus, E. multilocularis, E. oligarthrus* and *E. vogeli* (Rausch and Bernstein, 1972). Opposing views on the breeding system and population genetics of *Echinococcus* further complicated discussion on the taxonomy (Thompson and Lymbery, 1988). Over the past 50 years, however, field and laboratory observations have revealed considerable phenotypic variability among isolates of *Echinococcus*. This variation has largely been observed in *E. granulosus*, and between isolates of the parasite from different species of intermediate host (Table 1).

Based mainly on mitochondrial DNA-based studies, it has been shown that *E. granulosus* comprises 10 genotypes (G1 to G10), which have been elevated to distinct species, comprising *E. granulosus* sensu stricto (G1, G2 and G3); *E. equinus* (G4); and *E. ortleppi* (G5) and its sister species, *E. canadensis* (G6, G7, G8, G9, G10) (Nakao *et al*. 2007). Recently, the lion strain has been proposed as another new species, *E. felidis* positioned as a sister taxon of *E. granulosus* s.s. (Hüttner *et al*. 2008). *Echinococcus shiquicus*, a sister species to *E. multilocularis*, has been found in Tibet (Xiao *et al*. 2005; 2006). This article provides an overview of some of the phenotypic variation observed in isolates of *Echinococcus*, why the concept of a 'strain' was developed, and how molecular and other data have reinforced the need to revise the taxonomic status so that up to 9 species are now recognised.

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PHENOTYPIC VARIATION IN *ECHINOCOCCUS*

Early physiological and biochemical studies

Smyth and Smyth (1964) were the first to point out that the *Echinococcus* organisms have a mode of reproduction which favours the expression of mutants, with the result that new parasite variants or strains can readily arise. However, the key to our

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Table 1. Some of the phenotypic variation observed in *Echinococcus granulosus* (Modified from Thompson and McManus, 2002)

Morphological differences
– Hook number and dimensions (due to host effects)
– Strobilar dimensions (total adult worm length; 2–11 mm)
– Reproductive anatomy (e.g. 25–80 testes)
Developmental differences
Metacestode (e.g. cyst growth and protoscolex formation) and adult (e.g. growth and maturation), *in vitro* and *in vivo*
Differences in host infectivity and specificity
Experimental infections and epidemiological observations
Differences in chemical composition
Protein, carbohydrate, nucleic acids and lipids in metacestodes and adult worms
Differences in metabolism
Carbohydrate metabolism of metacestodes and adults
Differences in proteins
Electrophoretic separation; protein and isoenzyme analysis
Host–parasite relationship differences
Immunoreactivity and/or antigenicity – EG95 vaccine; antigenic differences between G1 and G6 genotypes

Note: DNA differences reflect this phenotypic variation.

understanding of the phenomenon of strain variation came in the early 1970s with the seminal studies of James Desmond Smyth on the strobilar development of *Echinococcus in vitro* where he demonstrated that in media which supported the development and maturation of *E. granulosus* of sheep origin, isolates of the parasite from horses failed to develop or mature (Smyth and Davies, 1974) (Fig. 1). This was at first attributed to suspected faults in technique or media components but when, after two years of experiments involving some 200 cultures, horse material failed to strobilate, it was realised that this result represented a new phenomenon, and it was concluded that isolates of *E. granulosus* from horses represented a different 'strain' from that of sheep with some unique (possibly nutritional) factor or culture condition for sexual differentiation (Smyth and Davies, 1974) which even today has not been identified. This appears to be some unusual requirement, for isolates of buffalo, camel, cattle, goat and human origin were subsequently shown to differentiate sexually in the (sheep) *in vitro* system (Macpherson and Smyth, 1985). In addition to the *in vitro* growth differences, epidemiological data (Hatch and Smyth, 1975; Smyth, 1977) suggested that the horse-dog form may be a different strain with possibly no, or only low infectivity to humans. Indeed, all subsequent studies have reinforced this view.

That sheep and horse hydatids represent different strains was confirmed by the demonstration of biochemical differences between them (McManus and Smyth, 1978, 1982). A comparison of the basic biochemical composition and carbohydrate metabolism of protoscolex larvae showed marked differences, providing a striking example of a phenomenon described for other helminths such as *Hymenolepis diminuta* and *Haemonchus contortus*. Protoscoleces of the horse strain contained less protein and RNA and more lipid than those of the sheep strain. Under aerobic conditions, the sheep strain used more oxygen and glycogen and produced more succinic and acetic acids but less lactic acid than the horse strain. Anaerobically, there was little difference in glycogen utilisation, but the sheep strain produced less succinic and lactic acids, and more acetic acid and ethanol. In other words, both aerobically and anaerobically, the major end-products of the sheep strain were mainly acetic acid with some succinic acid, whereas those of the horse strain were mainly lactic acid with some succinic acid. A subsequent, wider ranging study carried out on *E. granulosus* from Kenya involving protoscoleces from five different host species (sheep, goats, camels, cattle and humans) and adults, also indicated metabolic variability (McManus, 1981) although the significance of the variability was, at the time, unclear.

In other early work, isoenzyme markers were used to discriminate species and strains of *Echinococcus* (Le Riche and Sewell, 1978; McManus and Smyth, 1979; Macpherson and McManus, 1982). In one study, extracts of protoscoleces of the horse and sheep strains of *E. granulosus* and *E. multilocularis* were compared on the basis of their isoenzyme patterns for 10 enzymes (acid phosphatase, lactate dehydrogenase, malate dehydrogenase, malic enzyme, glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), isocitrate dehydrogenase, adenylate kinase, aldolase and alpha-glycerophosphate dehydrogenase) by means of isoelectric focusing (IEF) in polyacrylamide gels; interspecific and intraspecific differences were apparent in the isoenzyme profiles of all the enzymes except adenylate kinase, whose pattern and activity was identical for both strains of *E. granulosus* (McManus and Smyth, 1979).

The isoenzyme patterns for GPI and PGM were later compared by IEF for soluble enzyme extracts from protoscoleces obtained from hydatid cysts of human, camel, cattle, sheep and goat origin in Kenya (Macpherson and McManus, 1982). Consistent GPI and PGM isoenzyme patterns were obtained for larvae of human, camel and sheep material, with the camel material exhibiting distinct profiles for both enzymes. Two isoenzyme patterns were evident in the goat material; the more common goat patterns were similar to those of human, cattle and sheep material. The more rare goat patterns were similar to those obtained for the camel samples.

The molecular era and the genetic basis for the observed phenotypic variation in E. granulosus

In the 1980s the new techniques in molecular biology offered great potential for providing a new approach

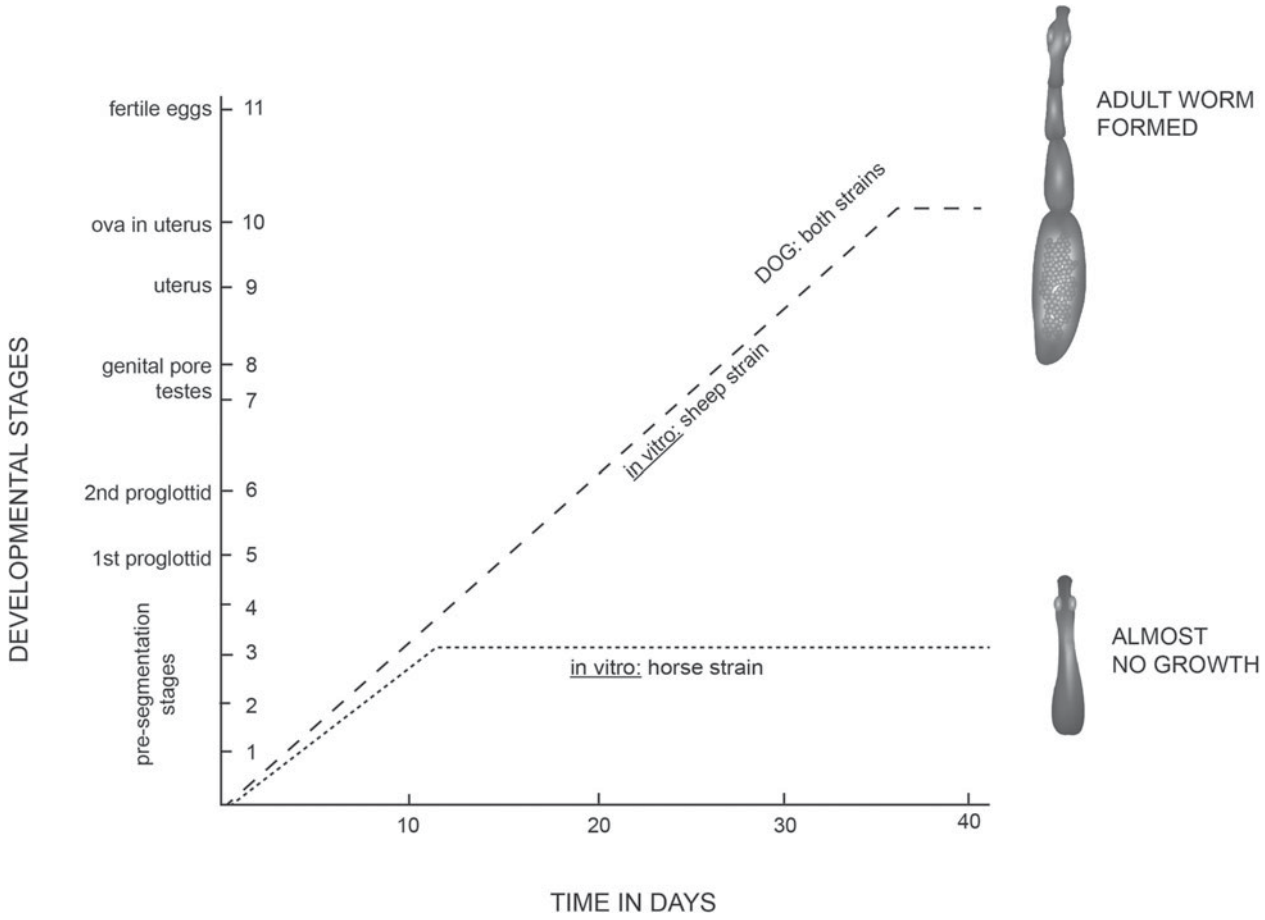

Fig. 1. Development of the protoscoleces of *Echinococcus granulosus* of sheep and horse origin under identical conditions *in vitro*. The sheep strain formed proglottids and grew to sexual maturity; the horse strain failed even to segment and did not develop beyond stage 3. In the most successful cultures, the rate of development *in vitro* of the sheep strain lagged only a few days behind that in the dog *in vivo*. (Modified from Smyth and Davies, 1974).

to studies on the taxonomy, genetics and population biology of parasites. The sensitivity and specificity of DNA analysis was ideal for ascertaining the extent of strain variation in *E. granulosus*. Indeed, the application of molecular tools for characterizing isolates of *Echinococcus* has had a major impact on our understanding of the population genetics, epidemiology and taxonomy of the parasite and the genetic basis of the phenotypic variation evident.

Having isolated high molecular weight and pure DNA from *E. granulosus* and *E. multilocularis* that was cleavable by restriction enzymes (McManus *et al*. 1985), McManus and Simpson (1985) used cloned DNA fragments of the ribosomal RNA gene of *Schistosoma mansoni* to discriminate between isolates of *E. granulosus* from UK horses and sheep as well as *E. granulosus* from *E. multilocularis* used restriction fragment length polymorphism (RFLP) analysis involving a Southern blot hybridization approach. Subsequently, a recombinant plasmid (coded pEG18) with a 2·3 kb DNA fragment unique for *E. granulosus* was cloned and used, with other cloned probes, in RFLP analysis to independently and reproducibly discriminate between the UK horse and sheep strains of *E. granulosus* and to characterize a large number of isolates from different host species from various geographical areas (McManus and Rishi, 1989).

The study did not demonstrate any significant genetic variation within the United Kingdom horse/dog or sheep/dog strains but confirmed the distinctiveness of the two strains shown previously. The sheep/dog strain was shown to be cosmopolitan in its distribution and fertile bovine material originating from the United Kingdom, Kenya, Spain and India conformed to this strain by DNA hybridization. In contrast, cattle isolates from Holland produced markedly different DNA hybridization banding profiles indicating that cattle could harbour more than one strain of *E. granulosus*. Similarly, it was shown that goats could harbour two different strains of *E. granulosus*, the sheep/dog strain and a form which infects camels. The analysis showed that the strain of *E. granulosus* infecting equines in Spain and Ireland was genetically identical to that found in horses in the United Kingdom. There was also a different strain infecting pigs in Poland and Yugoslavia. This pig/dog strain appeared to be very similar genetically to the forms of *E. granulosus* which use camels and goats as intermediate hosts and was

similar, though not identical, to the variant infecting Dutch cattle.

Building on this work, a procedure was developed that linked the polymerase chain reaction (PCR) with RFLP analysis of the ITS-1 region of ribosomal DNA of *E. granulosus*; this method (PCR-RFLP analysis) was used successfully to study variation in *E. granulosus* isolates from several regions (Bowles and McManus, 1993). An example of a study where the procedure proved useful was that of Wachira *et al.* (1993) who examined 208 larval isolates and 40 worm samples of *E. granulosus* from various hosts in Kenya, confirming the existence of the camel and sheep strains there and showing that the distribution of the camel strain appeared to be restricted to the Turkana region, where camels are kept as livestock. The study also showed that although the life-cycle patterns of the two strains overlap both geographically and in intermediate and definitive hosts, the strains maintain their homogeneous genetic identity.

A modification of the procedure, involving a specific and sensitive PCR/semi-nested PCR system, was later developed by Dinkel *et al.* (2004) for the rapid diagnosis of *E. granulosus* strains in East Africa. Most recently, a multiplex PCR for the simultaneous detection and typing of *E. granulosus* strains has been developed that has potential for worldwide application in large-scale molecular epidemiological studies on the *Echinococcus* genus (Boubaker *et al.* 2013).

The seminal *in vitro* culture studies of Smyth provided a platform of understanding for both earlier and subsequent observations on differences in the development and infectivity between isolates of the parasite from various host species in different parts of the world (Thompson and McManus, 2002). The realisation that variants of *E. granulosus* develop at different rates in the definitive host has meant that the timing of anthelmintic administration designed to remove worm burdens before patency should be reconsidered. Further, the fact that some variants appear to be poorly infective to humans has resulted in a reappraisal of the public health significance of *Echinococcus* in areas where such variants are endemic (Thompson and McManus, 2002).

Given the epidemiological significance of such intraspecific variation in *E. granulosus* and the international efforts to establish control programmes in different endemic regions, new nomenclature was needed to reflect the phenotypic variability evident between host-derived isolates of *E. granulosus* (Thompson and McManus, 2002). Thus, the concept of a 'strain', first coined by Smyth, was further developed as a result of the accumulating genomic information to describe variants that differ from other groups of the same species in gene frequencies or DNA sequences, and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis (Thompson *et al.* 1995; Thompson and McManus, 2002; McManus and Thompson, 2003).

Rapidly evolving mitochondrial (mt) sequences have provided a rich source of information for research in evolutionary biology, population genetics and phylogenetics. They have been used extensively in studies of *Echinococcus* since Bowles *et al.* (1992) first used the sequence of a region of the mitochondrial cytochrome c oxidase subunit I (CO1; *cox1*) as a marker of species and strain identity and as a preliminary indication of evolutionary divergence within the genus. Molecular studies, using mainly mtDNA sequences, have now identified ten distinct genotypes (G1–G10) within *E. granulosus*, a categorisation which follows closely the pattern of strain variation that has emerged based on biological characters (Eckert and Thompson, 1997; Thompson and McManus, 2002; Lavikainen *et al.* 2003; McManus and Thompson, 2003). These genotypes/strains of *E. granulosus* comprise: sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7), cervid strain (G8), pig/human strain (G9) and Fennoscandian cervid strain (G10).

PHYLOGENETIC ANALYSIS

An invaluable approach for investigating levels of divergence between taxa is by means of phylogenetic trees, a number of which have been constructed for *Echinococcus*, based on partial and complete mitochondrial DNA and nuclear DNA sequences (Bowles *et al.* 1995; Thompson *et al.* 1995; Le *et al.* 2002; Nakao *et al.* 2007, 2010; Moks *et al.* 2008; Saarma *et al.* 2009; Knapp *et al.* 2011).

These DNA-based phylogenetic studies have shown an even more pronounced genetic divergence between the ten *E. granulosus* genotypes than previously recognised and, taking this information and other criteria into account, the taxonomy of *Echinococcus* has been revised (Thompson and McManus, 2002; McManus and Thompson, 2003; Nakao *et al.* 2007; Thompson, 2008; Pednekar *et al.* 2009; Saarma *et al.* 2009; Knapp *et al.* 2011) (Table 2). *E. granulosus* is now considered as a complex consisting of at least four species: *E. granulosus* s.s. (genotypes G1 to G3), *E. equinus* (G4) and *E. ortleppi* (G5), but the species status of genotypes G6 to G10 is still ambiguous (Thompson and McManus, 2002; McManus and Thompson, 2003). Taxonomic revision to unify the cervid, camel and pig strains into a single species, *E. canadensis*, has been suggested (Lavikainen *et al.* 2005; Nakao *et al.* 2007; Moks *et al.* 2008).

DNA sequencing of mitochondrial genes and nuclear protein coding genes, phylogeny construction and morphological studies have identified *E. shiquicus* as a new sister species to *E. multilocularis* (Xiao *et al.* 2005, 2006, Nakao *et al.* 2007; Saarma

Table 2. Some taxonomic features of the genus *Echinococcus*

Species	Strain/genotype	Intermediate hosts	Known definitive hosts
Echinococcus granulosus (sensu stricto)	Sheep/G1	Sheep, cattle, pigs, camels, goats, macropods	Dogs, foxes, dingoes, jackals, hyaenas
	Tasmanian sheep/G2	Sheep, cattle	Dogs, foxes
	Buffalo/G3	Buffaloes, goats, cattle, sheep	Dogs, foxes
Echinococcus canadensis	Camel/G6	Camels, goats, sheep, cattle	Dogs
	Pig/G7	Pigs, wild boars, beavers, cattle	Dogs
	G9	Pigs	Dogs
	Cervid/G8 and G10	Cervids	Wolves, dogs
Echinococcus felidis	Lion	Warthogs, zebra, wildebeest, bushpigs, buffaloes, antelopes	Lions
Echinococcus equinus	Horse/G4	Horses, other equines	Dogs
Echinococcus ortleppi	Cattle/G5	Cattle, buffaloes, goats, sheep	Dogs
Echinococcus multilocularis	Minor isolate variation resulting in European, Asian and North American clades	Small mammals, domestic and wild pigs, dogs, monkeys	Foxes, dogs, cats, wolves, racoon-dogs, coyotes
Echinococcus shiquicus	None reported	Plateau Pika	Tibetan foxes
Echinococcus vogeli	None reported	Small mammals (pacas, agoutis, spiny rats)	Bush dogs, domestic dogs
Echinococcus oligarthrus	None reported	Small mammals (pacas, agoutis)	Wild felids (jaguars, pumas, cougars, ocelots, jaguarondis)

Note: All species, except *E. equinus*, have been shown to be infective to humans.

et al. 2009; Knapp *et al*. 2011). The larval form occurs in the plateau pika, *Ochotona curzoniae*, found in Shiqu County, in the Qinghai-Tibet plateau region of western Sichuan, China. The adult stage has been isolated from the Tibetan fox, *Vulpes ferrilata*. The metacestode develops into a unilocular cyst mainly in the liver. Its zoonotic transmission potential is presently unknown. Furthermore, recent mitochondrial DNA studies have identified *E. felidis* as a sister species to *E. granulosus* s.s. whose adult stage has been isolated from African lions with warthogs acting as intermediate hosts (Hüttner *et al*. 2008, 2009). There are no data available on the pathogenicity of *E. felidis* to humans, but its public health impact may be minimal, as lions are largely restricted to national parks and game reserves where there is little human activity. *E. felidis* may, however, have an impact on pastoralists in East Africa who coexist with wildlife (Moro and Schantz, 2009).

FINAL COMMENTS

There is remarkable genetic homogeneity within *E. multilocularis* specimens isolated from different geographical regions based on the analysis of classical coding and non-coding DNA targets although European, Asian and North American clades have been defined (Bowles *et al*. 1992; Haag *et al*. 1997; Rinder *et al*. 1997; Yang *et al*. 2005; Nakao *et al*. 2009). In contrast, a number of distinct genotypes of *E. granulosus* (designated G1-G10) are now recognised, with the genotype cluster G1-G3 (*E. granulosus* s.s.) and genotype G6 being responsible for the majority of humans infections.

E. granulosus is now considered as a complex consisting of at least four species having differing life-cycle patterns, host specificity, development rates, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents and pathology with important implications for the design and development of vaccines, diagnostic assays and drugs impacting on the control of hydatid disease (Thompson and McManus, 2002). This variability has practical relevance when considering the extensive genetic variability recently reported in the complete coding DNA sequence of the EG95 antigen of *E. granulosus* which may have a direct influence on host specificity and vaccine efficacy.

The oncosphere-expressed EG95 antigen is the basis of a recombinant vaccine developed for use in livestock to prevent infection with *E. granulosus* (Gauci *et al*. 2005). The EG95 antigen was originally cloned from the G1 genotype of *E. granulosus* and the protein has been found to be encoded by members of a small family of related genes in this genotype. Following on from an earlier study, which revealed substantial nucleotide substitutions (encoding amino acid substitutions) for EG95 in the G6/G7 genotypes (Chow *et al*. 2008), a recent study by Alvarez Rojas *et al*. (2012), used genomic DNA cloning techniques to characterize seven *eg95*-related gene fragments from the G6 genotype of *E. granulosus*. Three proteins appeared to be encoded by these genes. Considerable differences were found between the EG95 related proteins from the G6 genotype compared with the EG95 protein from the G1 genotype. These differences suggest that the EG95-related proteins from the G6 genotype may have different antigenic

epitopes compared with the current vaccine antigen. These data have implications for future vaccine design and provide information that would enable a G6 genotype-specific vaccine to be developed against *E. granulosus*, should this be considered a desirable addition to the available tools for control of cystic echinococcosis transmission.

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