Genetic and phenotypic analysis of Tunisian *Theileria annulata* clones

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(*Received 5 August 2002; revised 15 October 2002; accepted 15 October 2002*)

SUMMARY

Many parasite species are known to show high levels of genetic diversity, yet the consequences of this diversity for host–parasite interactions are not well understood. Variation in phenotypic traits such as growth rates and the ability to form transmission stages are raw material for natural and artificial selection to act upon with consequences for the evolution of the parasite species and disease control. In order to study genetic and phenotypic diversity amongst *Theileria annulata* parasites, a collection of 52 parasite clones was generated from cattle isolates and tick material recently collected in Tunisia. Genetic diversity was assessed using PCR-RFLP and monoclonal antibody markers, and genetically distinct clones selected for further study. Clones varied significantly in their growth rates in culture at 37 °C, their viability after a period of culture at 41 °C and their differentiation rates into transmission stages after culturing at 41 °C. The viability of a clone after culturing at 41 °C could not be predicted from its growth rate at 37 °C, but across clones, differentiation rates were positively correlated with growth rates at 37 °C. All 3 *in vitro* measures are likely to have relevance to parasite–host interactions in animals with clinical theileriosis, and should be acted on by within-host and between-host selection.

Key words: *Theileria annulata*, genotyping, phenotyping, growth rates, differentiation, transmission.

INTRODUCTION

Many, perhaps all, parasite species exhibit high degrees of genetic variability. With the increasing application of molecular biology methods (Groth & Weatherall, 2000) in both laboratory and field studies, a large body of data on this variability is now being built up (Thompson, 2000). Genetically distinct parasites of the same species can also vary in fundamental aspects of their biology such as growth rates and their ability to transmit (e.g. Mackinnon & Read, 1999; Davies, Webster & Woolhouse, 2001). Although the genetic basis of this variation is often not known, it has far reaching implications for virulence to the host, transmission rates between hosts and the evolution of parasite characteristics. If a range of different parasite growth rates exists in a population, host responses or artificial disease control methods will select those parasites which are best able to survive. If, for example, these are parasites with high growth rates, then an increase in disease severity in hosts may be expected.

In a natural infection of *Theileria annulata* in a cow, sporozoites from the tick vector invade host white blood cells inducing them to divide. Many cell types can be invaded, but only macrophages and B cells are immortalized, allowing them to replicate, potentially indefinitely (lymphoblastogenesis) (Campbell & Spooner, 1999). The asexually dividing multinucleate parasite stage, termed the macroschizont, hijacks the host cell division machinery and as the leucocytes divide, the parasites divide within them, producing an exponentially increasing infected cell population where the parasites never leave the host cells (Hulliger *et al*. 1964). Differentiation to the transmission stages of the parasite in culture occurs in response to elevated temperatures (Hulliger, Brown & Wiilde, 1966; Shiels *et al*. 1992), although the precise role of fever *in vivo* is less clear (Shiels, 1999). During differentiation, the asexual macroschizonts enlarge and the number of parasite nuclei increases several-fold until the host cell cytoplasm is almost full of parasite nuclei. Then parasites begin to transform into merozoites and more than a hundred very small nuclei per infected cell can be seen (Shiels *et al*. 1992). Eventually, the host cell bursts and individual merozoites are released. These infect red blood cells and develop into piroplasms, ready to be taken up by the tick vector. The life-cycle is well suited to *in vitro* culture systems, as the asexual parasites (macroschizonts) in their host cells replicate exponentially, requiring only culture medium supplemented with foetal calf serum and incubation at 37 °C with 5% CO_2. Culturing and cryopreservation methods for the parasite are well established (Brown, 1987) allowing large amounts of material to be generated easily. When culture temperatures are raised to 41 °C, the

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Parasitology (2003), **126**, 241–252.
DOI: 10.1017/S0031182002002810 Printed in the United Kingdom

parasites begin to differentiate towards the transmission stages, via the merozoite (Hulliger *et al*. 1966; Shiels *et al*. 1992).

The purpose of this study was to use PCR-based and mAb methods to distinguish genetically different *T. annulata* parasite clones derived from material recently collected in Tunisia. The total period of *in vitro* culture was kept to a minimum so that laboratory selection pressure was avoided as far as possible. Different parasite clones were then compared under standard *in vitro* laboratory conditions to assess variation in growth rates and their rates of differentiation into transmission stages.

MATERIALS AND METHODS

Parasite material and cloning methods

Theileria annulata isolates previously collected in Tunisia from cows and ticks and cryopreserved at CTVM were used (Ben Miled, 1993; Ben Miled *et al*. 1994). Parasite stocks isolated by Ben Miled *et al*. (1994) from cattle with clinical theileriosis in Northern Tunisia and numbered 20, 33 and 36 were chosen because previous genetic work had shown them to contain a complex mix of parasite genotypes (Ben Miled *et al*. 1994). Isolate cell cultures were diluted to give 1/3 infected cells per well of a 96-well plate. Cryopreserved ground-up-tick stabilate derived from ticks collected on 2 Tunisian farms, Bechir and Chergui (in the highly endemic Bouhanach area of Northern Tunisia) were used as a source of *Theileria* sporozoites. Stocks 20, 33 and the tick-derived stabilates Bechir and Chergui were all collected in the semi-arid region of Northern Tunisia, with stock 36 collected further south in the sub-humid region of Northern Tunisia (Ben Miled, 1993). The 2 tick stabilates were separately seeded onto 2 bulk preparations of leucocytes from a single uninfected British Friesian cow at a series of dilutions (from 1 : 10 to 1 : 1000) in 96-well plates. The lowest dilution producing expansion of the bovine cells was chosen to maximize the probability that individual bovine cells were infected with a single sporozoite.

Once individual infected cells began to divide, cultures were named with the prefix B for Bechir, C for Chergui or the number of the cow from which they were derived, and given an individual identifying number. Clones were gradually bulked up and as soon as cell densities allowed (average 29 days), 30 ml of passage 2 culture in exponential growth was cryopreserved. This minimized the impact of *in vitro* selection on the cultures. All methods were standard (Brown, 1987) and culture medium was RPMI 1640 (PAA laboratories) supplemented with 20% foetal calf serum (PAA laboratories), 2·0 mM L-glutamine (PAA laboratories) and 1 × antibiotic/antimycotic solution (Sigma, final concentrations of 100 units penicillin/ml, 0·1 mg streptomycin/ml and 0·25 μg amphotericin B/ml). Cell lines were cryopreserved in culture medium with 10% dimethyl sulfoxide.

PCR-RFLP analysis

Cell pellets (approximately 10^7 infected cells) derived from cultures in exponential growth were washed 3 times in PBS, and then stored at −20 °C. DNA was extracted using a standard phenol : chloroform technique (Maniatis, Fritsch & Sambrook, 1982). Two polymorphic *T. annulata* antigen genes, *Tams-1* (Shiels, Katzer & D'Oliviera, 1997*b*) and *Spag-1* (Katzer *et al*. 1994), were used to genotype the parasite clones. Primers used for *Tams-1* were 17a (forward, 5′ ATG CTG CAA ATG AGG AT 3′) and 20 (reverse, 5′ GGA CTG ATG AGA AGA CGA TGA G 3′c) (Kirvar *et al*. 2000), and those for *Spag-1* were Spag3 (forward, 5′ GCG GTA TCA GTA TCA GGT GAC TC 3′) and Spag1b (reverse, 5′ TTG CAC CAG CGA TTT GAC CTC 3′). PCRs were carried out in 25 μl volumes with a final concentration of 1 μM each primer, 0·2 mM of each dNTPs, 1·5 mM $MgCl_2$, 1 × buffer, 1 unit of Promega *Taq* polymerase in storage buffer B and 150 ng of infected cell DNA. Amplification of the *Tams-1* product was carried out at 94 °C for 5 min, then 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by a final extension time of 10 min at 72 °C. For the *Spag-1* product the extension time was 2 min 15 sec per cycle.

The product sizes were 770 bp for *Tams-1* and between 1400 and 1700 bp for *Spag-1*. Both products were digested with *Rsa1*, and the *Tams-1* product was also digested with *Taq1*. Digestions were carried out in volumes of 20 μl with 5 units of enzyme, 1 × buffer, 1 × BSA and 5 μl of PCR product, and incubated at 65 °C (*Taq1*) or 37 °C (*Rsa1*) for 2 h. Then 10 μl of the digestion reaction was run out on a 2% agarose gel with TAE and the banding pattern analysed. Alleles were considered different if their general banding patterns differed, small differences in the sizes of each band due to differences in the overall PCR product size of *Spag-1* were ignored. This will have lead to an underestimate of the number of different alleles.

Monoclonal antibody analysis

The clones originating from the tick collections were also analysed using mAbs. Infected cells from cultures in exponential growth were washed twice and resuspended in PBS. Cytospin preparations were made with 25 μl samples of cultures, and stored desiccated at −20 °C. Two murine primary mAbs were used to examine variability among antigens of the parasite clones. MAb 7E7 was raised against the macroschizont-infected cell line *Ta*Tu 270/1 (Ben Miled *et al*. 1994) and mAb 1C7 was raised against *Ta* Hissar-infected lymphocytes (Shiels *et al*. 1986*b*), and are *T. annulata*-infected cell specific, but their precise

https://doi.org/10.1017/S0031182002002810 Published online by Cambridge University Press

molecular targets have not been defined. Slides were warmed to room temperature whilst still desiccated, and acetone-fixed at −20 °C for 15 min. Samples were incubated with 10 μl of primary antibody (culture supernatants; 1C7 used neat, 7E7 at 1 : 10 dilution) at room temperature in a humid chamber for 30 min. Wells were washed 3 times with PBS, and incubated with 15 μl of a FITC-labelled anti-mouse IgG secondary antibody (1 : 50 dilution, Diagnostics Scotland) with 2 μg/ml DAPI, again for 30 min. Wells were washed 3 times with PBS, before a coverslip was mounted with 50% glycerol, 2·5% DABCO in PBS. Fluorescence was examined under a UV microscope fitted with filters for FITC and DAPI. Bovine cell and parasite nuclei were clearly identifiable with the DAPI filter, and the presence or absence of FITC fluorescence of the parasite was determined.

Analysis of growth rates at 37 °C

Twelve clones derived from the tick stabilates (therefore in cells of identical bovine genotype) with different parasite genotypes were selected to determine variation in the growth rates of cultures maintained at 37 °C. Two replicate 2 ml cultures for each clone were set up with an initial density of 1×10^5 live cells/ml in a 24-well plate. On each of the next 4 days, cells were resuspended and 40 μl samples taken from each culture at exactly 24 h intervals for analysis. The medium was not changed over this time-period. Three replicates were carried out.

Live cell density was determined by FACScan analysis. The 40 μl culture sample was added to 400 μl of FACS flow diluent. Just prior to analysis, 40 μl of fluorescent beads of a known concentration around 1×10^6 beads/ml (Flow-count™ Fluorospheres from Coulter Corporation) and 40 μl of 260 mg/ml propidium iodide was added. FACS scanner settings are shown in Table 1.

Plotting FL2 against FSC readings, the FACS scanner separates the beads, and the live, dead and apoptotic cell populations. The beads fluoresced most strongly, but dead cells, and to a lesser extent apoptotic cells, fluoresced due to propidium iodide uptake. The apoptotic cell population was confirmed using an Annexin-V-FLUOS Staining kit (Roche Molecular Biochemicals). From the ratio of the count of beads to live cells and the bead concentration, the live cell density was calculated.

Differentiation rates at 41 °C

For 11 of the 12 clones for which growth rates were determined, the ability to differentiate into merozoites (i.e. to produce transmission stages) in culture was determined (the stock culture of clone C1 died). In each replicate, 2 cultures were established for each clone and 3 replicates were performed (total of 6 cultures for each clone). Seven millilitre cultures were set up with $2{\cdot}5 \times 10^5$ live infected cells/ml in 25 cm^2 flasks and maintained at 41 °C. Cultures were examined daily, the flasks turned upright, the cells allowed to settle, and 3 ml of medium replaced. If cell density had increased significantly, dilution of the cells to around $2{\cdot}5 \times 10^5$ cells/ml was carried out. Preliminary studies had suggested that prolonged culture at 41 °C caused many cultures to die, so after 5 days the cultures were returned to 37 °C for a further 2 days (3 ml medium replaced daily). Cytospin samples were taken from all cultures on day 7 to assess differentiation rates by both Giemsa stain and IFAT. A period of 5 days and 41 °C is sufficient for the parasite to become committed to an irreversible phase of differentiation which can continue at 37 °C, but will have reduced the proportion of cells differentiating relative to that obtained by continuous culture at 41 °C for 7 days (Shiels *et al*. 1992).

Table 1. FACScan settings for flow cytometry to assess growth rates

Detector	Voltage	Amplitude gain	Mode	Threshold
FSC	E00	1·4	Lin	52
SSC	242	1·0	Log	
FL1	560	1·0	Log	
FL2	366	1·0	Log	
FL3	366	1·0	Log	

From Giemsa-stained slides, at least 1000 infected cells were examined and separate counts made of (a) live cells containing normal macroschizonts, (b) live cells containing enlarged macroschizonts (with many nuclei and filling the whole of the host cell's cytoplasm), (c) merozoites within host cells or recently burst out from them, and (d) dead cell remains. Viability estimates were made based on the proportion of live cells (a + b + c/a + b + c + d) and differentiation rates of live cells were based on the proportion of differentiated cells (b + c/a + b + c).

For cultures with high enough viabilities (proportion of cells alive greater than 0·25), a polyclonal rabbit antiserum was also used to assess differentiation by IFAT. This antiserum was raised against a *T. annulata* rhoptry (*TamR1*) fusion protein and allows an assessment of the proportion of parasite-infected cells producing the rhoptry protein and therefore fully committed to development into merozoites (Shiels *et al*. 1994, 1997*a*). The protocol was similar to that used on undifferentiating cultures using 15 μl of a 1 : 200 dilution of the antiserum, and 15 μl of a 1 : 150 dilution of a FITC-labelled anti-rabbit IgG secondary antibody (Diagnostics Scotland) with 2 μg/ml DAPI. Slides were mounted with fluorescent mounting medium (DAKO Corporation) and at least 900 cells counted with each scored as giving a positive or negative signal with the antiserum.

https://doi.org/10.1017/S0031182002002810 Published online by Cambridge University Press

Statistical analysis

Analysis was carried out using Splus 2000 (Data Analysis Products Division, 1999).

As a fixed amount of nutrient was supplied to cultures in the growth rate experiment on day 0, the number of live cells is expected to follow a logistic curve over time. The live cell density was plotted against time (days 1–4) and the following function fitted to the data for each culture:

$$y = \frac{A^{rx}}{(1+A^{rx})/k} \quad \text{where} \quad A = \frac{C_0}{1-(C_0/k)}.$$

The value for C_0 (the initial density of live cells) was fixed at 1×10^5 cells/ml and time, x, was measured in days. The remaining 2 parameters, r, the intrinsic growth rate, and k, a measure of density-dependent reduction of growth rates, were estimated separately for each culture using non-linear least squares regression.

For 5 cultures a k value could not be fitted to the data, and the cell density data were found to fit an exponential growth curve well. In these cases, r was calculated assuming exponential growth:

$$y = e^{rt}C_0,$$

and no k value was assigned to the culture.

The derived r values were then analysed using ANOVA using Type III sums of squares to improve the handling of missing values. In total, 5 cultures were excluded completely from the analysis because of fungus contamination towards the end of the 4 day culture period (1 in replicate 1, 3 in replicate 2 and 1 in replicate 3). The significance of the interaction term was tested first, before the main effects were tested in a model without the interaction term. *Post-hoc* analysis of differences between groups was carried out using the Bonferroni method (Data Analysis Products Division, 1999).

Data on viability of cultures, and the proportion of live cells which had differentiated on day 7 were collected for 11 clones. Analysis of viability was performed for all 11 clones, but because all 6 clone C2 cultures showed very low viability, differentiation rates could only be analysed for the remaining 10 clones. For the summary graphs, the proportion of live cells, and the proportion of differentiated cells for each culture were arcsine transformed, then mean and standard errors calculated for each clone from the normalized data. These values were then back-transformed and plotted as proportion data. For the formal analysis, generalized linear models with a binomial error structure were fitted to the cell count data and as residual variation was overdispersed for both analyses, the significance of model parameters was assessed using F ratios. Again, the significance of the interaction term was tested first, before the main effects were tested in a model without the interaction term.

Differentiation rates as determined by the antiserum were correlated with the rates as determined by Giemsa-staining in a generalized linear regression model with binomial errors. The proportion differentiating by the Giemsa method was arcsine transformed in order to normalize it before it was used as an independent variable. The proportion of cells alive and the differentiation rates determined by Giemsa staining were first arcsine transformed to normalize them before being correlated to the growth rate data using Pearson's product-moment correlation. A simple jack-knife type analysis was used to confirm that C3 was an outlier in the analysis correlating differentiation rates against growth rates. The data point for each clone was removed in turn, and the analysis repeated. This showed that excluding clone C3 lead to a significant association between the variables, but all other exclusions did not (t_7 always $<1{\cdot}19$, P always $>0{\cdot}27$).

RESULTS

Clones generated for this study

A collection of 52 clones of *T. annulata*-infected bovine leucocytes was prepared. Sporozoites from tick-derived stabilates were used to generate 28 clones *in vitro* using bovine leucocytes from a single cow of British origin. From 3 stocks collected from clinical cases in Tunisia (20, 33 and 36) a total of 24 further clones was generated by limiting dilution of the original cow cells (7, 10 and 7 from each case respectively). These clones were taken to represent combinations of single parasite genotypes and cow cells which could have arisen naturally in the field and are hereafter referred to as parasite clones.

At the start of genotyping and growth rate analysis experiments, all clones had undergone 3 passages (about 5 weeks) of *in vitro* culture. The differentiation experiment was started with parasites of passage 5 (about 7 weeks in culture).

Genotyping

PCR products were derived from 2 antigen genes, *Tams-1* and *Spag-1*. Across all parasite clones, the size of the *Tams-1* product was identical (770 bp), but clones exhibited polymorphism in the size of the *Spag-1* products from 1400 to 1700 bp (data not shown). No clone produced more than 1 *Spag-1* PCR product, providing evidence of clonal populations. *Tams-1* products were digested with *Taq1* and *Rsa1*, and *Spag-1* products with *Rsa1* only. Fig. 1 shows a selection of the alleles of the *Spag-1*/*Rsa1* digests. Banding patterns were grouped into alleles based on spacing (slight differences in band sizes due to variation in the PCR products for *Spag-1* were ignored). This led to a conservative estimation of the genetic variation amongst the clones. Table 2 shows the full

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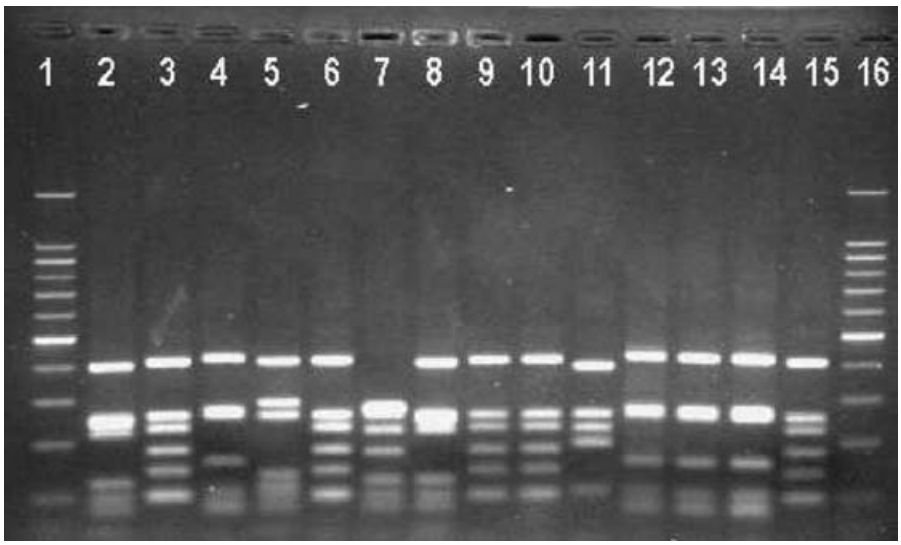

Fig. 1. *Spag-1* PCR products digested with *Rsa1*. Lanes 1 and 16: 100 bp marker, lanes 2 to 15: clones 20-8, 33-8, 36-7, 20-5, 33-1, 20-2, 20-3, 33-4, 33-9, 20-9, 36-3, 36-4, 36-6, 33-3 respectively. The allele numbers for lanes 2 to 15 are 3, 6, 4, 3, 6, 2, 3, 6, 6, 7, 4, 4, 4, 6 respectively.

results for the 3 sets of alleles analysed. Across the 52 parasite clones, there were 6 *Tams-1/Taq1* alleles, 4 *Tams-1/Rsa1* alleles, 10 *Spag-1/Rsa1* alleles and combining all three, 22 different genotypes were differentiated. For the collection of clones made from the tick stabilates (28 parasite clones), a total of 15 different genotypes were differentiated, and for the 24 clones derived from cow isolates, a total of 8 different genotypes were distinguished.

Monoclonal analysis

The 28 parasite clones derived from tick stabilates were also screened with mAb 7E7 and 1C7, and each clone scored as positive or negative for each. Within a clone, the percentage of cells reacting positively with the antibody was either 100% or 0% (data not shown), providing evidence that clonal parasite populations had been established.

Across the clones, all 4 possible combinations of positive and negative results for the 2 mAbs were found (Table 1). Combining these results with those from the genotyping meant that out of the 28 tick-derived clones tested, 22 different genotypes could be discriminated.

Growth rates at 37 °C

A scatter plot of the raw data from the FACScan analysis is shown in Fig. 2. From the ratio of beads (from a stock of known concentration) to live cells, live cell densities over time were calculated (Fig. 3). Intrinsic growth rate (r) values were calculated for each culture, assuming logistic growth and analysed by ANOVA. A significant interaction ($F_{22,31}=2{\cdot}57$, $P<0{\cdot}01$) was found between replicate and clone and significant differences ($F_{2,53}=60{\cdot}03$, $P<0{\cdot}01$) were found across replicates, however significant ($F_{11,53}=9{\cdot}53$, $P<0{\cdot}01$) differences in growth rates were still found among clones. Thus, although growth rates varied across the replicates, once these had been controlled for, there were still strong differences among clones (Fig. 4). Values of r varied from 0·85 (clone C8) to 1·56 (clone C6). Based on exponential growth without nutrient limitation, these values correspond to cell doubling times of 19·56 and 10·67 h.

Clones were then divided into fast, medium and slow growing groups, based on cut off values of $r=1{\cdot}25$ and $r=1$ (Fig. 4). After replicate differences had been controlled for, these groups showed highly significant differences in their growth rates ($F_{2,53}=47{\cdot}48$, $P<0{\cdot}001$) as expected, and not surprisingly there was no additional significant effect when clone was added to this model ($F_{9,53}=1{\cdot}10$, $P>0{\cdot}10$). Growth rate group explained over 90% of the variance explained by clone, and using *post-hoc* analysis it was found that all 3 groups differed significantly ($P<0{\cdot}05$) from each other (Fig. 4).

Viability and differentiation rates at 41 °C

Cloned cell lines were subjected to 5 days of culture at 41 °C, followed by 2 days at 37 °C. Cytospin samples were prepared on day 7, and stained with Giemsa. Parasites which have made the commitment to form transmission stages are seen as either enlarged macroschizonts as more mature merozoites. At least 1000 infected cells were examined and separate counts made of (a) live cells containing normal macroschizonts, (b) live cells containing enlarged macroschizonts (with many nuclei and filling the whole of the host cell's cytoplasm), (c) merozoites within host cells or recently burst out from them, and (d) dead cell

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Table 2. Full genotyping results for the 52 clones

(T and S refer to the PCR products from the *Tams-1* and *Spag-1* genes respectively. Columns 1C7, 7E7 and mAb give the results using the 2 mAbs separately and together. The final column shows the complete genotype based on the 5 measures analysed.)

Source	Clone	T-*Taq1*	T-*Rsa1*	S-*Rsa1*	Genotype	1C7	7E7	mAb	Combined genotype
Bechir	B1	1	1	1	1-1-1	+ve	+ve	1-1	1-1-1-1-1
Bechir	B2	1	1	2	1-1-2	−ve	+ve	0-1	1-1-2-0-1
Bechir	B3	1	1	1	1-1-1	−ve	+ve	0-1	1-1-1-0-1
Bechir	B4	2	3	4	2-3-4	−ve	+ve	0-1	2-3-4-0-1
Bechir	B5	1	1	2	1-1-2	+ve	−ve	1-0	1-1-2-1-0
Bechir	B6	1	1	5	1-1-5	−ve	−ve	0-0	1-1-5-0-0
Bechir	B7	2	2	5	2-2-5	+ve	−ve	1-0	2-2-5-1-0
Bechir	B8	1	1	3	1-1-3	−ve	+ve	0-1	1-1-3-0-1
Bechir	B9	2	2	3	2-2-3	−ve	−ve	0-0	2-2-3-0-0
Bechir	B10	3	1	5	3-1-5	−ve	−ve	0-0	3-1-5-0-0
Bechir	B11	1	1	2	1-1-2	−ve	+ve	0-1	1-1-2-0-1
Bechir	B12	3	1	5	3-1-5	−ve	−ve	0-0	3-1-5-0-0
Bechir	B13	1	1	1	1-1-1	−ve	−ve	0-0	1-1-1-0-0
Bechir	B14	1	1	2	1-1-2	−ve	+ve	0-1	1-1-2-0-1
Bechir	B15	2	2	5	2-2-5	+ve	−ve	1-0	2-2-5-1-0
Bechir	B16	3	1	2	3-1-2	+ve	−ve	1-0	3-1-2-1-0
Bechir	B17	1	1	2	1-1-2	−ve	−ve	0-0	1-1-2-0-0
Bechir	B18	1	1	2	1-1-2	−ve	+ve	0-1	1-1-2-0-1
Bechir	B19	2	2	3	2-2-3	+ve	−ve	1-0	2-2-3-1-0
Chergui	C1	4	4	3	4-4-3	+ve	+ve	1-1	4-4-3-1-1
Chergui	C2	4	4	8	4-4-8	+ve	−ve	1-0	4-4-8-1-0
Chergui	C3	3	1	9	3-1-9	−ve	+ve	0-1	3-1-9-0-1
Chergui	C4	4	4	3	4-4-3	+ve	−ve	1-0	4-4-3-1-0
Chergui	C5	3	1	3	3-1-3	+ve	+ve	1-1	3-1-3-1-1
Chergui	C6	3	1	1	3-1-1	−ve	+ve	0-1	3-1-1-0-1
Chergui	C7	4	4	8	4-4-8	+ve	−ve	1-0	4-4-8-1-0
Chergui	C8	4	4	1	4-4-1	−ve	−ve	0-0	4-4-1-0-0
Chergui	C9	3	1	1	3-1-1	+ve	+ve	1-1	3-1-1-1-1
Cow 20	20-2	6	4	2	6-4-2				
Cow 20	20-3	3	1	3	3-1-3				
Cow 20	20-4	5	3	3	5-3-3				
Cow 20	20-5	5	3	3	5-3-3				
Cow 20	20-6	3	1	3	3-1-3				
Cow 20	20-8	3	1	3	3-1-3				
Cow 20	20-9	5	4	7	5-4-7				
Cow 33	33-1	1	1	6	1-1-6				
Cow 33	33-2	1	1	6	1-1-6				
Cow 33	33-3	1	1	6	1-1-6				
Cow 33	33-4	1	1	6	1-1-6				
Cow 33	33-5	1	1	6	1-1-6				
Cow 33	33-6	1	1	6	1-1-6				
Cow 33	33-7	2	2	10	2-2-10				
Cow 33	33-8	1	1	6	1-1-6				
Cow 33	33-9	1	1	6	1-1-6				
Cow 33	33-10	1	1	6	1-1-6				
Cow 36	36-1	1	4	4	1-4-4				
Cow 36	36-2	1	4	4	1-4-4				
Cow 36	36-3	5	4	4	5-4-4				
Cow 36	36-4	5	4	4	5-4-4				
Cow 36	36-5	5	4	4	5-4-4				
Cow 36	36-6	5	4	4	5-4-4				
Cow 36	36-7	5	4	4	5-4-4				

remains. Viability estimates were made based on the proportion of live cells (a+b+c/a+b+c+d) and differentiation rates of live cells were based on the proportion of differentiated cells (b+c/a+b+c).

Viability varied across replicates and clones, and the interaction between these was significant ($F_{20,33}=4{\cdot}86$, $P<0{\cdot}01$). Viability varied significantly ($F_{2,53}=7{\cdot}97$, $P<0{\cdot}01$) with the replicate, but when this was controlled for, there was still a highly significant ($F_{10,53}=15{\cdot}22$, $P<0{\cdot}001$) effect of clone. So, despite differences across replicates, clones were shown to vary in their viability after 5 days of

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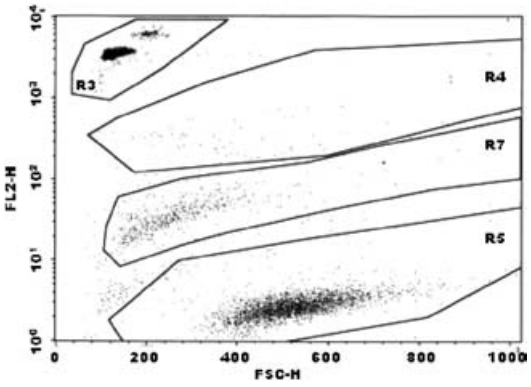

Fig. 2. Typical scatter graph output of FL2 versus FSC from the FACScan. The regions outlined represent R3: fluorescent beads of known concentration; R5: live cells; R4: dead cells; R7: apoptotic cells.

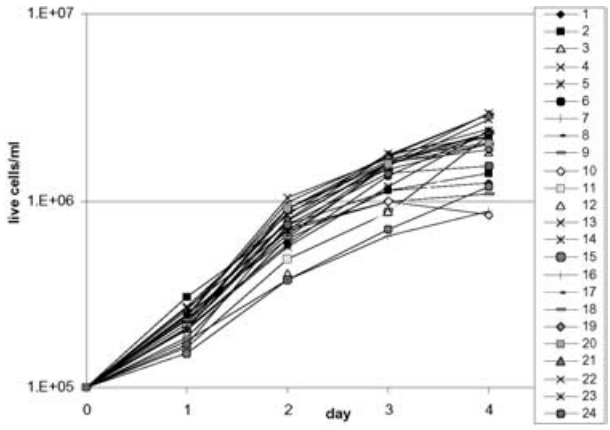

Fig. 3. Plot of live cell densities over days 0 to 5 for individual cultures.

culturing at 41 °C (Fig. 5). Despite stock cultures at 37 °C continuing to grow normally over the same period, all 6 replicate cultures of clone C2 died before day 7 of the experiment. Clone B5 also showed low viability at day 7.

Across clones, there was no significant correlation ($t_9 = 1{\cdot}16$, $P > 0{\cdot}10$, graph not shown) between viability after 7 days and growth rate at 37 °C, suggesting that a clone's growth rate at 37 °C was not a predictor of its ability to tolerate higher culturing temperatures.

From a total of 66, 50 cultures had a viability on day 7 over 25%, and data from these were used to assess differentiation rates (the proportion of live cells which had differentiated to enlarged macroschizonts or merozoites). Differentiation rates varied with replicate and clone, and the interaction term was significant ($F_{15,23} = 8{\cdot}89$, $P < 0{\cdot}01$). Differentiation rates varied significantly ($F_{2,38} = 25{\cdot}01$, $P < 0{\cdot}01$) with the replicate but when this was controlled for, there was still a highly significant ($F_{9,38} = 45{\cdot}01$, $P < 0{\cdot}001$) effect of clone. So, despite differences across replicates, clones were shown to vary in their differentiation rates (Fig. 5).

Differentiation rates measured using the anti-rhoptry antiserum were plotted against those obtained from the Giemsa-stained smears (data not shown) and the best fit line was found to have a gradient of 0·79 and an intercept of 0·02. The gradient was below 1 as expected if some of enlarged macroschizonts seen in the Giemsa-stained slides were not yet producing the rhoptry protein picked up by the antiserum. This relationship suggests that 79% of all parasites seen to be differentiating on the basis of Giemsa-stained slides were producing the rhoptry protein. When these 2 measures were formally analysed, the correlation was highly significant ($F_{1,49} = 444{\cdot}27$, $P < 0{\cdot}001$, $R^2 = 0{\cdot}89$).

A practical problem was encountered with highly differentiating cultures. Parasites which had differentiated to merozoite stages were often found grouped together, around the burst host cell remains. However, in cultures with high rates of differentiation, single merozoites were often seen scattered over the slide and not associated with their host cell remains. For these cultures it is likely that some cells counted as dead had in fact already differentiated into merozoites and consequently high differentiation rates were underestimated.

Correlation between differentiation rates at 41 °C and growth rates at 37 °C

When differentiation rates (as determined from the Giemsa-stained slides) were plotted against growth rates at 37 °C, a general positive trend across clones was found, but this was not significant ($t_8 = 1{\cdot}06$, $P > 0{\cdot}10$). However, it was clear that clone C3 was an outlier, and when this was removed, the association became significant ($t_7 = 2{\cdot}85$, $P < 0{\cdot}05$, correlation coefficient = 0·733, Fig. 6). Differentiation rates were also calculated for the same groups of clones as in the growth rate analysis (but excluding clone C3). These points also fell within the association across the individual clones (Fig. 6). Thus clones with faster growth rates at 37 °C were found to have higher differentiation rates at 41 °C.

DISCUSSION

The majority of studies on *Theileria* parasites have been based on uncloned parasite isolates. As selection occurs among parasite genotypes, it is the characteristics of these genotypes which are relevant to studies of parasite variation, and mixed isolates are inappropriate. A collection of cloned *T. annulata* parasites was therefore generated for this study, for which the opportunity for selection *in vitro* had been minimized. Selection for cloned infected cell lines which could grow *in vitro* could not be avoided. However, it is improbable that differences in phenotype were created during the production of clones, in fact the opposite is expected. The characteristics of

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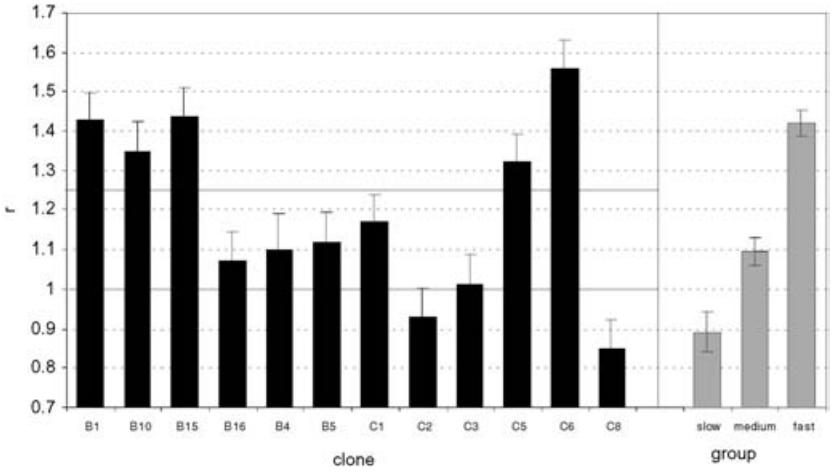

Fig. 4. Growth rates of clones at 37 °C. Data are means and S.E. for each clone (black bars) or group (grey bars).

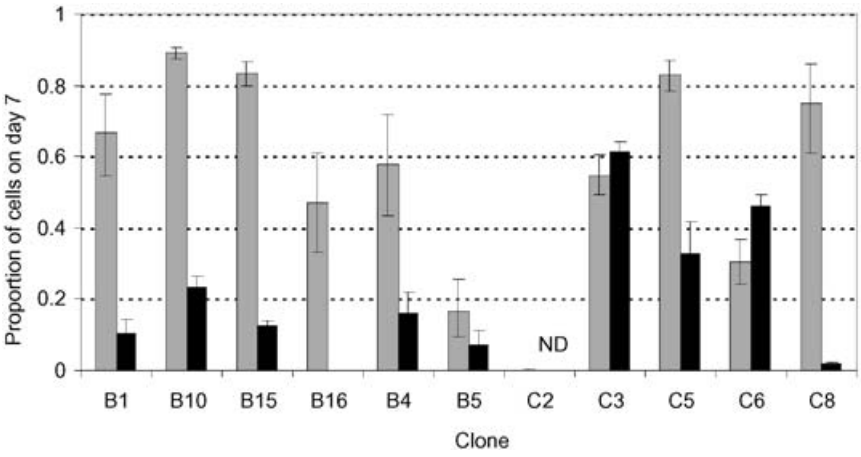

Fig. 5. Viability (grey bars) of clones and differentiation rates (solid bars) after 5 days of culturing at 41 °C and 2 days at 37 °C. Data are means and S.E. for each clone. ND, Not done.

parasite clones found in this study are therefore taken to be representative of variation that could have been found in natural infections.

A variety of methods, including mAb (Bishop *et al.* 1993*b*; Pinder & Hewett, 2001), isoenzyme analysis (Allsopp, Gibson & Stagg, 1985), oligonucleotide probes to parasite DNA (Conrad *et al.* 1987; Allsopp *et al.* 1989; Bishop *et al.* 1993*b*; Bishop, Spooner & Sohanpal, 1996), arbitrary-primed PCR and RAPDs (Bishop, Sohanpal & Morzaria, 1993*a*; Sparagano, Zanaa & Ambrose, 1998), minisatellites (Bishop, Morzaria & Gobright, 1998), PCR-RFLP (Geysen *et al.* 1999) and sequencing (Toye *et al.* 1995; Collins & Allsopp, 1999) have been used to demonstrate genetic variation in *Theileria parva*. The number of studies genotyping *T. annulata* is more restricted, but methods used include isoenzyme analysis (Melrose *et al.* 1984; Ben Miled *et al.* 1994; Martin-Sanchez & Garcia-Fernandez, 1999), mAb (Shiels *et al.* 1986*a*; Ben Miled *et al.* 1994), oligonucleotide or nucleic acid probes (Ben Miled *et al.* 1994; Shiels *et al.* 1995), RAPDs (Martin-Sanchez & Garcia-Fernandez, 1999), and sequencing (Katzer *et al.* 1994, 1998; Shiels *et al.* 1995; Gubbels *et al.* 2000). The advantage of the PCR-RFLP method used here for analysing DNA from cloned parasites is its speed, allowing a large number of clones to be genotyped in a relatively short time. The genotypic data collected were based on only a few, mostly antigen, genes. This represents only a small fraction of the parasite genome, so whilst they have been used to show that two clones are different, they cannot be used to show that two clones are the same. However, because of the complex banding patterns that would be generated, it is far less suitable for the analysis of mixed infections, or uncloned isolates.

Levels of genetic diversity found in field studies of *T. parva* seem variable, but can be very high (Bishop *et al.* 1994; Geysen *et al.* 1999). However, the numbers of isolates included in these studies were small. The present study found high levels of genetic diversity. Using 3 sets of PCR-RFLP markers and 2 mAbs, 22 genotypes could be discriminated out of the collection of 28 clones derived from tick material. It is possible that using more markers would have allowed the discrimination of all 28 clones into

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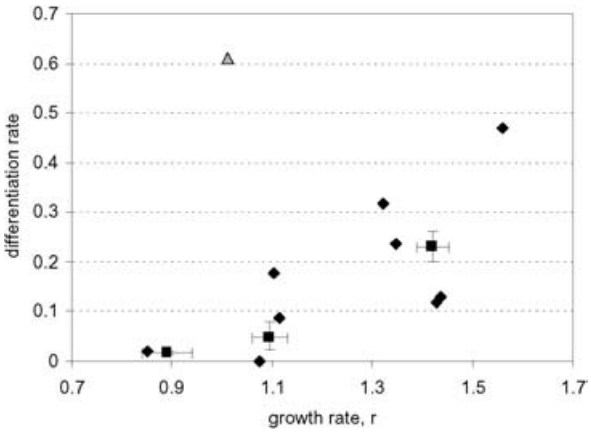

Fig. 6. Correlation between differentiation rates and growth rates at 37 °C. Individual clones (except clone C3) are represented by black diamonds, clone C3 by the black triangle and growth rate groups by grey squares. Error bars on the group data points are 1 S.E.

distinct genotypes. The high genetic diversity found is consistent with previous studies of the same collection of parasites from Tunisia (Ben Miled *et al*. 1994; Katzer *et al*. 1998) and a more geographically widespread collection (Gubbels *et al*. 2000), but the number of studies based on large numbers of parasite isolates or sequences from different geographical locations is still too low to say whether *T. annulata* populations are always genetically diverse.

The variation in reactivity to the mAb suggests that the clones could elicit different immune responses by the host. Although antibody responses to macroschizont-infected cells are not thought to be protective in *T. annulata* (Boulter & Hall, 2000), antibodies generated by the presence of one clone in a host could potentially affect the growth rates of other clones in the same host.

Phenotypic characteristics of *Theileria* parasites cannot of course be attributable just to the parasite, as the bovine host cell must have a role in infected cell population dynamics. In the growth rate and differentiation experiments, all clones analysed were parasite-infected leucocytes from the same individual cow, so bovine cell genotype was controlled for. Further work is necessary in order to determine the exact bovine cell phenotype infected in each case. However, these clones represent actual parasite–host cell combinations that could have arisen from genetically distinct sporozoites infecting a host in the field and, as such, phenotypic differences between them represent variation that could be acted on by selection.

There has been no attempt in this study to correlate the genetic differences found with phenotypic characteristics measured. There is no expectation that differences in the genes analysed should lead to differences in complex characteristics such as growth and transmission rates. Thus, the genotyping work was to exclude clones that may have been genetically identical before an assessment of the extent of phenotypic differences was started.

Growth rates have been shown to differ across clones or isolates of several protozoan parasites, including *Babesia bovis* (Nevils *et al*. 2000) and *Plasmodium falciparum* (Chotivanich *et al*. 2000) *in vitro*, and *Plasmodium chabaudi* (Mackinnon & Read, 1999), *Trypanosoma brucei gambiense* (Diffley *et al*. 1987), *Schistosoma mansoni* (Davies *et al*. 2001) and *Plasmodium yoelli* (Knowles & Walliker, 1980) *in vivo*. For *Theileria*, a few estimates of parasite-infected cell growth rates in culture have been made (e.g. Hulliger, 1965; Hooshmand Rad, 1975) but factors such as the presence of uninfected cells and varying culture media make their interpretation difficult. The only controlled study to date comparing cloned *T. annulata* growth rates in culture at 37 °C found 2 cloned cell lines increased by 6·7-fold and 4·2-fold over a 48 h period (Shiels *et al*. 1992).

Several very difficult studies have attempted to quantify replication rates of *T. parva* in cattle (Jarrett, Crighton & Pirie, 1969; Radley *et al*. 1974; Morrison *et al*. 1981). These studies suggest that replication rates may be dose dependent and more complex than can be described with a simple exponential. As these authors point out, calculations of growth rate *in vivo* are complicated by host effects such as immunity and can thus only measure net growth rates in the face of an unquantifiable rate of destruction of parasitized cells.

The present study attempted to quantify and compare growth rates of several different genotypes of *T. annulata* parasites in bovine cells under standard culture conditions. All clones went through an identical cloning procedure and period of *in vitro* culture before the analysis began. Growth rates were very sensitive to initial starting conditions, but highly repeatable given the same starting conditions (hence the significant replicate and clone : replicate interaction terms), and clear differences in growth rates were found between clones. Assuming exponential growth, the growth rates found here correspond to cell doubling times of between 10 and 20 h. These represent intrinsic growth potentials of the clones, and serve as a means for comparing clones. They do not represent the overall changes in cell density directly observed from cultures (including those used here) where at least some nutrient limitation occurs.

The relevance of these differences to the *in vivo* situation requires experimental investigation. If growth rates differ *in vivo* as *in vitro*, 2 predictions can be made. First, since the clonal expansion of macroschizont-infected host cells and the resultant tissue damage is thought to give rise to the main pathology in *T. annulata*, clones with higher growth rates should produce more severe disease in cattle. Second, slower growing clones would be expected to lose out in competitive interactions in mixed infections.

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The survival of cultures for a period of 5 days at 41 °C was also variable, some clones maintained high viability despite the raised temperatures whilst others were killed by the same treatment. Such differences between clones may be due to differences in the activity of proteins such as heat shock or other proteins (Shiels, 1999), and could not be predicted from their growth rates at 37 °C. However, if the viability differences found in this experiment correspond to different abilities of the clones to survive in a host undergoing a fever response, this would predict strong selection of temperature-resistant parasite clones during fever *in vivo*.

Differentiation rates were highly variable across clones, ranging from close to 0% for B16 parasites to over 60% for C3 parasites. The results using the antiserum raised against the *T. annulata* rhoptry antigens correlated well with those from the Giemsa-stained slides, but with a gradient of less than one. Around 20% of parasites seen to be differentiating on Giemsa-stained slides did not give a positive signal with the anti-rhoptry antiserum, as expected if this threshold had not yet been passed. Importantly, differentiation rates seem to be independent of viability at 41 °C, demonstrating that differentiation is an active progressive process, and not simply a response to poor environmental conditions. There was no evidence that clone C2 cultures died because of rapid rates of transformation at 41 °C.

Differences in differentiation rates for *T. annulata* have been reported previously, but for a more limited collection of clones (Shiels *et al.* 1992) and similar differences in the production of transmission stages between clones has been documented for *P. chabaudi* (Mackinnon & Read, 1999) and *S. mansoni* (Davies *et al.* 2001) *in vivo*.

Generally, differentiation rates at 41 °C correlated positively with growth rates at 37 °C. However, clone C3 was a notable exception to this pattern and in a previous study, differentiation rates were higher for the slower growing of 2 clones (Shiels *et al.* 1992). Further clones should be tested in order to establish how robust this correlation is. The 2 days of culturing at 37 °C after the period at 41 °C in the differentiation experiment has the potential to confound the correlation between differentiation rates and growth rates at 37 °C. Once returned to 37 °C, undifferentiated parasite-infected cells of clones with faster growth rates should replicate faster, thus potentially reducing the estimated proportion of cells differentiated by a larger degree than for clones which grow slower at 37 °C. However, the effect found here, a positive correlation between the two variables, is the opposite expected from confounding of the variables. If such confounding effects have occurred in this experiment, it suggests that the real correlation between differentiation rates at 41 °C and growth rates at 37 °C is more strongly positive than the statistics here show.

If the *in vitro* results found in these experiments also apply in the *in vivo* situation, the expectation is that parasites with faster growth rates in an animal without fever would also produce more transmission between hosts when the animal suffers fever. This would be consistent with evolutionary hypotheses that virulent pathogens are selected for because of their transmission advantage (Levin, 1996). The relationship between growth rate and transmissibility has not been well studied in parasites, but was found to be positive across clones of *P. chabaudi* in mice (Mackinnon & Read, 1999) and *S. mansoni* in mice (Davies *et al.* 2001). Similar correlations have been shown for a wider range of infectious agents, but importantly not all fit this general rule (Lipsitch & Moxon, 1997). If slower growing parasites are less competitive in susceptible cattle at both within-host growth and between-host transmission, then the persistence of extensive variation in these traits is harder to explain. However, *T. annulata* also infects hosts such as endemic cattle breeds and Asiatic buffalo, where few if any clinical signs are expressed. It is possible that selection for parasite characteristics in these hosts acts as a form of balancing selection, maintaining a parasite population with a wide range of growth rate and differentiation rates which are then able to infect susceptible breeds.

Duncan Brown and Erol Kirvar provided very helpful advice at the outset of this project, and Gwen Wilkie provided initial training in the culturing and cloning techniques. The authors are very grateful to Leila Ben Miled and Mohammed Darghouth for the original collection of material this study is based upon. Dan Haydon, Darren Shaw, Louise Matthews, Loeske Kruuk and Margo Chase-Topping have provided mathematical and statistical advice at various times. Mark Woolhouse has made helpful comments throughout the project and on the manuscript. The work was supported by a Wellcome Trust Veterinary Fellowship (ref no. 054156) to L. T.

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