Sub-species typing among bovine *C. parvum* isolates by PCR-PAGE using a novel microsatellite + telomere primer scheme

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Abstract A PCR-PAGE procedure based on a novel primer set including a previously described microsatellite (M) plus a telomere anchor (+T) was used to biotype *C. parvum* isolated from bovine sources in three categories: geographically diverse, intra herd, and specifically defined. The PAGE patterns resulting from the procedure consisted of from 8 to 18 bands in the 300 to 3,000 bp size range. Application of the procedure revealed widely divergent PAGE banding among the geographically diverse samples. Consistent banding patterns were observed among multiple calf hosts from the same dairy herd sampled over 1 to 2 year periods. Some differences were observed in PAGE banding between samples in different time periods although all independent samples in each sampling period (up to 5 shown) were homogeneous. Analysis of DNA from samples of the Iowa defined strain of *C. parvum* isolated in 1998 and in 2000 from the same source (University of Arizona) resulted in PAGE banding that was homogeneous. The sub-type differentiation effectiveness of the M+T PAGE procedure was observed to be substantial. Reference to work being prepared for publication elsewhere indicates that it is similarly effective for Type 1 samples.
Keywords *Cryptosporidium*; PCR; sub-species differentiation; telomere

Introduction

Typing of *Cryptosporidium parvum* at the sub-species level has several important potential uses. Among them are epidemiological investigations and their environmental analogues. Ultimately, the ability to differentiate among *C. parvum* isolated from various sources will contribute to understanding which types are associated with virulence, infectivity, pathogenicity and environmental persistence problems of importance to human health and animal husbandry.

Significant effort has been devoted to this problem by many researchers taking a wide range of approaches based on available technology. Techniques that have been applied to sub-species typing of *C. parvum* have included: western blotting (Nina *et al.*, 1992; Petersen *et al.*, 1992); pulse field gel electrophoresis (Piper *et al.*, 1998); multilocus enzyme electrophoresis (Spano *et al.*, 1998; Feng *et al.*, 2000); mutation scanning methods (Gasser *et al.*, 2001); DNA and RNA gene sequencing (Morgan *et al.*, 1999), RT-PCR (Stinear *et al.*, 1996), and RAPD (Morgan *et al.*, 1995).

The capability of virtually all procedures published to date, with the exception of ones sequencing-based, is limited to distinguishing between two main types, according to convention: Type 1, found until recently exclusively in the human population; and Type 2, that has been isolated from both human and animal hosts. The most widely applied procedures today are PCR-based microsatellite amplifications using either single or multiple locus primers to provide differentiation (Morgan *et al.*, 1999). These procedures are relatively simple and require equipment within the cost range of most practicing and research laboratories.

Sequencing-based procedures involve equipment and procedures that are significantly more demanding and costly, limiting the range of applicability, or at least the rate of data production. Recent application of this type of procedure has demonstrated its ability to

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identify a dozen or more *C. parvum* subtypes in the Type 2 category (Xiao *et al*., 2000). Although the approach has not yet been demonstrated to show diversity among Type 1 isolates, it has been shown to differentiate between *C. parvum* from animal host species (Xiao *et al*., 1999). Epidemiological data, however, have recently shown that the previously regarded human-specific genotype 1 group may also cause infection in other hosts (Morgan *et al*., 1999; O'Donoghue *et al*., 1999; Fayer *et al*., 2000).

The potential of the currently described genotypes to cause different clinical symptoms is unknown and even less is understood of differences in infectivity (Chappell *et al*., 1996; Finch *et al*., 1993).

Several requirements for molecular based typing and identification schemes have been identified as desirable for greatest utility. Protocols should be relatively straightforward and provide information on genetic linage and relationship of isolates. The qualities of the ideal detection system need to be applicable to clinical and environmental samples with similar sensitivity. In a diagnostic laboratory the assay needs to be reproducible, robust, and practical to apply. The needs for application of subspecies typing information thus dictate several criteria that can be used to screen different approaches. Desirable or essential criteria include:

- ability to differentiate between isolates of human and of animal origin;
- ability to produce sufficient information to distinguish between genetically different isolates;
- ability to produce stable and reproducible information on individual isolates;
- ability to conduct the procedure on a wide range of clinical and environmental sample types;
- procedural characteristics rendering the methodology accessible and practical to apply.

We have developed a novel procedure based on a PCR-PAGE microsatellite approach using a previously described locus (M1, Morgan *et al*., 1995), but we have added a 3′-end primer "anchor" in the telomere region. The approach was selected to meet the above criteria. Details of the procedure and its performance are described elsewhere (Blasdall *et al*., 2001). Here, we report on the protocol as applied to describing the characteristics of *C. parvum* found in three groups of sources having different potential for genetic interrelation. The groups were: (1) samples from diverse geographical origins; (2) samples from a single large (3,500) herd of dairy cattle (Holstein-Friesian) sampled periodically over a four year period; and (3) samples of the "Iowa" strain of *C. parvum* obtained from the University of Arizona between 1998 and 2000.

Methods

Isolates

In this study a total of 44 isolates were examined (i.e. oocysts were isolated from 44 independent faecal samples, each from a different animal) (Table 1). Three groups of isolates were included: (a) five isolates of widely spaced geographical origin; (b) isolates from a single dairy operation in the southwestern Sydney metropolitan area (SydSW); and (c) commercially available Iowa strain representatives. The geographically separate isolates included four representatives, one each from four sources in Australia and the Iowa strain for comparison. The four Australian sources included: commercial dairies in the southwest of Sydney (SydSW), the northwest of Sydney (SydNW) (approximately 30 km from SydSW), and in central New South Wales (NSWC) (about 400 km west of Sydney; and a European isolate (Bel) obtained through a colleague in the Netherlands. Samples for the second group were collected from the SydSW source in regular sampling between the beginning of 1998 and the first quarter of 2001. The Iowa isolates were obtained commercially from the University of Arizona in separate batches in 1998 and 2000.

Faecal samples

All Australian isolates were collected as fresh faecal samples. Samples were collected typically from 30–40 calves in the 7–30 day age range in a morning of field work on approximately bimonthly sampling trips. IFA stained smears of each sample were screened the same day. Over the succeeding day or two, oocysts were isolated from positive samples by Sheathers followed by Percoll (Sigma, St. Louis) cleanup (Ongerth and Stibbs, 1987). Specimens were stored at 4°C without preservative. Oocysts were typically stored fresh or formalin (1% v/v) fixed to preserve DNA accessibility.

Oocyst identification

Oocysts to be used for typing were examined by immunofluorescence staining using *Cryptaglow* monoclonal antibodies (Waterborne, New Orleans) (Ongerth and Stibbs, 1987). Oocysts were stained by DAPI/PI to permit counting nuclei (Slifko *et al.*, 2000). No evidence of *C. andersoni* has been found in either adults or juveniles of the Australian sources over the sampling period.

Oocyst disruption and DNA extraction

DNA was isolated from 10–50,000 nucleic acid containing oocysts, by manufacturer's instructions, using the QIAmp DNA mini Kit (Qiagen, Sydney).

PCR amplification using Microsatellite 1 and Telomere (+T) primers

All DNA isolates were amplified with primers Microsatellite 1 (M1) (5′ $(CAGA)_4$ 3′) and Telomere primer (GCCTAAACCTAAA) (Patent application pending). PCR amplification was performed in 40 µl volumes with 2 µl of DNA in PCR buffer, 4 mM $MgCl_2$, 250 µM of each dNTP, 10 pmoles of each primer, and 2 units of DNA Polymerase (Red Hot *Taq* polymerase from Integrated Sciences, Sydney). The tubes were subjected to 94°C for 3 min, then 30 cycles of 92°C for 20 s, 40°C for 20 s, and 60°C for 2 min, followed by a final extension at 60°C for 5 min. Positive and negative controls were included in each batch of tests. The positive control was the same 1998 SydSW isolate, to ensure reproducible and comparable fingerprints. A 10 µl aliquot of PCR product was visualised by electrophoresis on 1% agarose, 100 µg ethidium bromide gels. A second 5 µl aliquot of the PCR products were analysed by electrophoresis in 8% PAGE (Gradipore, Sydney, AU) and, post electrophoresis, gels were stained with 100 µg ethidium bromide for 15 minutes. All gels were recorded using UV transillumination and Polaroid Type 667 film, digitally captured using an EDAS system which consists of a DC290 digital Kodak camera and 1D Image analysis software290 (Life Technologies, Sydney, AU). Typing using the M1 microsatellite alone was used to provide discrimination between Type 1 and Type 2 samples. The M1-only PAGE banding patterns of all samples in this study were identical.

Table 1 Listing of isolates used, including sampling location, collection dates, and number of samples providing oocyst isolates. All isolates were *C. parvum* of bovine origin

Sample location	Date collected	Number of isolates
SydSW (Sydney)	Sept. 1988	12
SydSW (Sydney)	June 1999	6
SydSW (Sydney)	Aug. 2000	6
SydSW (Sydney)	April 2001	6
SydNW (Sydney)	Jan. 1999	4
NSWC (New So. Wales)	Feb. 1999	4
Bel (Europe)	Nov. 1999–Feb. 2000	2
Iowa – A (Arizona)	Sept. 1998	2
Iowa – A (Arizona)	Oct. 2000	2

Results and discussion

Geographically diverse sources

Application of the M+T typing procedure to bovine *C. parvum* from geographically diverse sources consistently revealed widely divergent PAGE banding patterns in the 300–3,000 bp range (Figure 1). The degree of differentiation seen in Figure 1 has been consistently observed among representatives of both Type 1 and Type 2 isolates from over 120 independent host sources from Australia, the USA, and Britain, including 74 from animals (cattle, sheep, goats, kangaroo) and 60 from humans (Blasdall *et al.*, 2001). Identical patterns have been observed in logical groupings of both human and animal sources. However, no identical patterns have yet been found in oocysts from logically unrelated samples (i.e. geographically diverse).

Homogeneous source samples

Application of the M+T typing procedure to bovine *C. parvum* from a homogeneous source resulted in a predominantly consistent pattern, but included significant differences over an extended (3–4 year) sampling period (Figure 2). Multiple oocyst batches isolated from different calves, and from which DNA was isolated at different times, when amplified using the M+T primers produced consistent PAGE banding. For example, oocysts from three calves sampled in April of 1998 (Lanes 1–3, Panel A, Figure 2), and in October of 1998, (Lanes 4–8, Figure 2), were produced identical banding. Two additional samples from October 1998 (Lanes 2 and 3, Panel B, Figure 2), were identical to the Panel A samples. A change in the PCR protocol to favour amplification of smaller fragments accounted for the different appearance of Panel A products and those in Lanes 2–3 of Panel B. The DNA of two independent samples from calves of the same SydSW herd collected in 1999 (Lanes 2–3, Panel B, Figure 2) were identical but exhibited a PAGE pattern different from the two sets of 1998 samples. The DNA from two additional sets of samples collected in 2000 and 2001 (Lanes 6–7 and Lanes 8–9, Panel B, Figure 2) were all identical. Clearly, the *C. parvum* infecting the calves of this large (3,00 milking head) dairy herd showed remarkably little diversity. Changes over time in the dominant isolate are still being investigated.

Over the course of the 18 months between running of the two gels shown in Figure 2 (Panel A and Panel B) developments and minor alterations to the PCR protocol had been

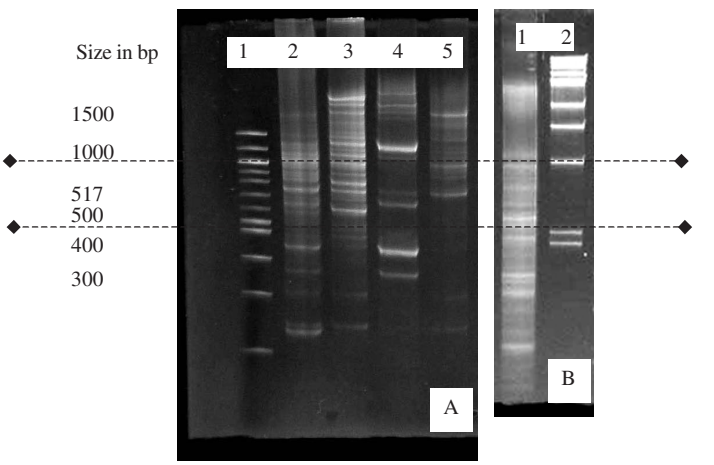

Figure 1 PCR using M+T primers, of isolates from four diverse locations, plus the Iowa strain. Note Panel A: Lane 1, 100 bp size standard (New England BioLabs, Brisbane, AU); Lane 2, SydSW 1998; Lane 3, Nth Sydney Basin 1998; Lane 4, Central NSW 1998; Lane 5, European 1999. Panel B: Lane 1, Iowa – A 2000; Lane 2, 100 bp size standard (New England BioLabs, Brisbane, AU)

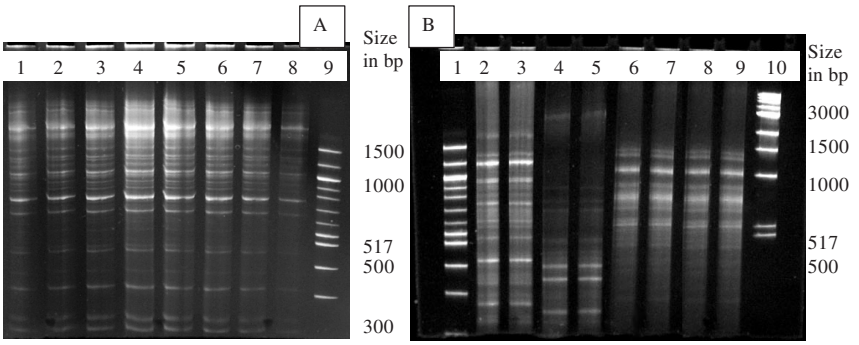

Figure 2 PCR, of isolates 24 months from SydSW location, using Microsatellite–Telomere primers SydSW isolates from 1998–2001. Panel A (8% PAGE run in November 1999): Lane 1, SydSW April 1998; Lane 2, SydSW April 1998; Lane 3, SydSW April 1998; Lane 4, SydSW Oct 1998; Lane 5, SydSW Oct 1998; Lane 6, SydSW Oct 1998; Lane 7, SydSW Oct 1998; Lane 8, SydSW Oct 1998; Lane 9, 100 bp size standard (New England BioLabs, Brisbane, AU). Panel B (8% PAGE run in August 2001): Lane 1, 100 bp size standard (New England BioLabs, Brisbane, AU); Lane 2, SydSW Oct 1998; Lane 3, SydSW Oct 1998; Lane 4, SydSW 1999; Lane 5, SydSW 1999; Lane 6, SydSW 2000; Lane 7, SydSW 2000; Lane 8, SydSW 2001; Lane 9, SydSW 2001; Lane 10, 1 Kb size standard (New England BioLabs, Brisbane, AU)

made. This resulted in different banding intensities of the two gels although careful examination revealed that the same bands were present. The later PCR conditions have been adopted in continuing work and are the ones described in the Methods section, above. Our interpretation of the data is that they clearly show that all samples from the same source at the same time were of the same genetic type. Considering the diversity illustrated in Figure 1 along with the consistency illustrated in Figure 2, the procedure offers a very substantial degree of differentiation while exhibiting stable characteristics. Each of the samples from independent sampling times were consistent. All samples from two separate 18 month periods (1998 and 2000–2001) were also completely consistent. Samples from the three different time periods could be distinguished, although the differences were only slight in comparison to differences observed among solely bovine-origin samples of geographically diverse origin. The relatively small differences observed between the 1998, 1999, and 2000–01 samples suggests the possibility that the telomere lengths of a single dominant isolate may have shortened leading to a shift in pattern between samplings. However, evidence from samples six months apart in 1998 and between 2000 and 2001 indicate that this isolate did not experience dramatic change over a moderate time period.

If telomere length may have shortened in proportion to effective passage cycling, and if an homogeneous isolate is being effectively passaged among the calves born to the herd, then the husbandry conditions provide a means of estimating the effective cycle time. Husbandry among large dairy herds appears to differ little. Calves born are kept in a common calving pen for as much as 48 hours during which they are fed largely colostrum. They are then transferred to individual pens that are typically in multiple long back-to-back double rows. Each calf will have limited opportunity for direct contact (through pen slats) with three neighbours, except for end pens having only two contact neighbours. Calves at the SydSW dairy are typically kept in the initial pen for about 30 days, then transferred by age group to another area of the pen complex. New calves will then occupy the pens after routine cleaning (sweeping, hosing down, and clean bedding) on approximately a 30 day cycle. Periodic sampling (ca. bimonthly over five years) has shown *C. parvum* infection prevalence in this herd ranging from 60–100%, averaging ca. 75% (Ongerth, unpublished data).

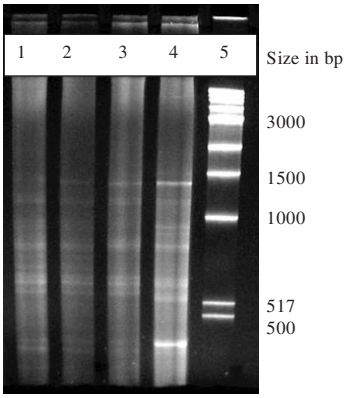

Figure 3 PCR, of isolates comparison over 24 months using Microsatellite–Telomere primers. Note: Lane 1, Iowa – A, 1998; Lane 2, Iowa – A, 1998; Lane 3, Iowa – A, 1998; Lane 4, Iowa – A, 2000; Lane 5, 100 bp size standard (New England BioLabs, Brisbane, AU)

Samples of a defined isolate

Samples of the Iowa *C. parvum* isolate were obtained from a single commercial source (University of Arizona) in September 1998 and in 2000. The DNA from three independent samples from 1998 and another from 2000 were analysed by the M+T procedure. The PAGE banding patterns obtained from these samples were consistent (Lanes 1–4, Figure 3).

Conclusion

Sub-species identification of *C. parvum* bovine isolates was demonstrated by application of our novel PCR-PAGE procedure that uses primers consisting of a microsatellite along with a 3′ telomere anchor. Effective fingerprints were produced from samples of three types including widely spaced geographical sources, multiple hosts at the same dairy herd location both in samples from the same date and on sample dates extending over a four year period, and from samples of a defined and passage-propagated isolate. The telomere repeat primer used was the most common reported telomere repeat of *C. parvum* (Liu *et al.*, 1998). On its own, this oligoneucleotide primer provided no genotyping information. However, it is when it is combined with a microsatellite within PCR-accessible range that the differentiation power is achieved. Discrimination between Type 1 and Type 2 *C. parvum* was provided by amplification using M1 alone.

Reproducible fingerprints were achieved for each isolate (up to ten, with five shown here) taken from the same location on the same sampling date. The fingerprints observed in samples from SydSW appeared to change over the full four-year period of observation. Comparison to samples of the defined "Iowa" strain over a comparable period (two years) showed no change. Examination of individual isolates over a longer period may shed light on the long-term stability of the target region of the *C. parvum* genome. Based on results reported here, the differentiation provided by the primer set in combination with apparent gene region stability would make the procedure useful for epidemiological investigation of outbreaks and related infections.

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