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Guidelines to Authors
Dear editor,

Hepatitis B virus (HBV) genotypes differ in the rate of seroconversion to anti-HBe,\textsuperscript{[1]} selection of core promoter,\textsuperscript{[1]} disease progression\textsuperscript{[2]} and response to treatment.\textsuperscript{[1]} Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)\textsuperscript{[3]} is one of the most widely used HBV genotyping techniques.

We studied 122 HBV DNA positive, chronic hepatitis B patients referred to the Clinical Virology Department from the Gastroenterology Department, Christian Medical College, Vellore. One hundred and five HBV DNA positive ‘healthy’ blood donors from John Scudder Memorial Blood Bank, Christian Medical College, were also studied. We used the earlier described PCR-RFLP for determining HBV genotypes.\textsuperscript{[3]} This technique involves the amplification of surface gene using primers P7 and P8, which flanked nucleotide positions 256 to 796. The PCR product was digested with \textit{Hinf I} and 5 U of \textit{Tsp509 I}, and digestion products were analysed in a 3\% agarose gel by electrophoresis.

Among the 227 subjects studied, HBV genotypes could be assigned for 215 subjects as previously described.\textsuperscript{[4]} However, in 12 (5.3\%) samples, the digestion pattern obtained did not match with any described pattern. Various investigators who used PCR-RFLP found a small proportion of their strains ‘untypeable’.\textsuperscript{[1,3,4]} To the best of our knowledge, none of the investigators studied this phenomenon further. We, therefore, studied the nucleotide sequences of these ‘untypeable’ strains with a view to identify altered/novel restriction sites.

Nucleotide sequencing was performed after amplification using primers P7 and P8.\textsuperscript{[3]} Sequencing was carried out using the ABI PRISM 310 genetic analyzer (PE Applied Biosystems, CA, USA). The nucleotide sequences of these 12 ‘untypeable’ samples were assigned HBV genotypes using the BLAST (basic local alignment search tool) (http://www.ncbi.nlm.nih.gov/BLAST/) programme. Eight samples were identified as genotype D, while the other four samples belonged to genotype C. The nucleotide positions of restriction sites and the digestion patterns of ‘untypeable’ samples are shown in the Table. We have described five novel digestion patterns, three for genotype D and two for genotype C.

We have analysed the putative digestion patterns of 54 complete genome/surface gene sequences from India available with the NCBI database using NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/index.php). None of the Indian sequences we analysed matched with any of the five novel patterns of digestion described in this study. It is, therefore, possible that the availability of fewer Indian HBV sequences

### Table: Nucleotide positions of restriction sites and the novel digestion patterns of 12 HBV strains ‘untypeable’ by PCR-RFLP

| Genotype | Nucleotide positions of \textit{Hinf I} sites | Digestion pattern with \textit{Hinf I} | Nucleotide positions of \textit{Tsp509 I} sites | Digestion pattern with \textit{Tsp509 I} | Novel digestion pattern\textsuperscript{a} | No. of samples |
|----------|------------------------------------------|--------------------------------------|---------------------------------------------|----------------------------------------|----------------------------------------|--------------|
| D        | 481 488 529 542 633 646                  | 354 480 589 602 633                  |                                            |                                        | D7          | 1            |
| D        | – + – – – – – 293,233,15                | – + + – – + 173,164,109,44,36,16     |                                            |                                        | D7          | 1            |
| D        | + – – – – – 300,226,15                  | – – + – + 282,164,43,36,16           |                                            |                                        | D8          | 6            |
| D        | ± – – – – – 526,300,226,15             | – ± + – + 282,173,164,109,43,36,16   |                                            |                                        | D9          | 1            |
| C        | – – + – – 274,148,104,15               | – – + – – 282,207,36,16              |                                            |                                        | C4\textsuperscript{b} | 3            |
| C        | – – – + – 287,148,104,15               | + – – + – 248,207,47,36,16           |                                            |                                        | C5\textsuperscript{c} | 1            |

The region of the HBV surface gene between nt. 256 and 796 was amplified and a 541 bp PCR product was obtained; \textit{Hinf I} sites were included in the antisense primer (P8) for nt. 781 (7); \textit{Tsp509 I} sites were included in sense primer for nt. 272 (P7) and present in all analysed sequences at nt. 307 (7); \textsuperscript{a}The digestion patterns in this table are numbered in continuation of those described in the original study (7); \textsuperscript{b}The sample with pattern C4 had a mixture of strains with guanine/adenine at nt. 480 creating a \textit{Hinf I/Tsp509 I} site, respectively; \textsuperscript{c}The sample with pattern C5 had an insertion of 13 bases and yielded a 554 bp PCR product

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in the NCBI database contributed to a poorer representation of the sequence variability from this geographical region, leading to the inadequate choice of restriction enzymes by investigators who developed this technique.

Genotype C was detected in 12 subjects by PCR-RFLP, and four ‘untypeables’ were further identified as genotype C after nucleotide sequencing. Therefore, it is possible that one in every four genotype C samples from the subcontinent may yield an ‘untypeable’ pattern, leading to an underestimation of infection with genotype C. The two novel patterns of digestion for genotype C described in this study could, therefore, be particularly useful for investigators using this technique for genotyping Indian strains. Studies from Southeast Asia show that genotype C is associated with more aggressive liver diseases\(^2,4\) and lower response rates to interferon alpha.\(^1\) Underestimation or failure to detect infection with genotype C may, therefore, have direct implications on patient management and treatment algorithms. Genotype D is found universally. The three novel digestion patterns that we have described for genotype D could hence be encountered with strains from any part of the world. The users of this genotyping technique globally may encounter ‘untypeable’ patterns.

As clinical differences among HBV genotypes are becoming increasingly relevant, there is a need for genotyping methods with high throughput. PCR-RFLP is an excellent technique for genotyping HBV, although a small proportion of strains is ‘untypeable’. The five novel digestion patterns described in our study may assist investigators using this technique resolve such ‘untypeable’ samples, thereby saving on the need to use labour-intensive and expensive options, such as nucleotide sequencing.

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Acute Urticaria Associated with Dicrocoelium dendriticum Infestation

Dear editor,

A 21-year-old Afghani woman, who had recently come to Germany, presented with acute urticaria affecting the complete integument. Parasitologic examination of four stool specimens obtained over two weeks revealed *Dicrocoelium dendriticum* eggs (Figure) and non-pathogenic amebae. Topical prednicarbate treatment and praziquantel (3 × 600 mg for 3 days) were recommended. Due to her state as refugee, the patient was lost to follow-up. Adult *D. dendriticum* live in the gall bladder and bile ducts of their final hosts (ruminants). Worm eggs are passed in faeces, which are swallowed by terrestrial snails. Snails excrete cercaria in mucous balls, which are eaten by ants. Infected ants stick to the tip of a grass-blade due to tetania of their mandibles. Herbivorous animals may ingest infected ants while grazing. In these animals, flukes develop, which migrate into the liver.\(^1\) Humans may rarely get infected by ingesting ants. However, in most cases, *D. dendriticum* eggs in human stools are not due to infection but by the ingestion of undercooked liver of infected animals.\(^2,3\) Such a “spurious” infestation (pseudoparasitism) seems unlikely in our patient, since *D. dendriticum* eggs were present in spite of a liver-free diet. Parasite-associated chronic urticaria are well known. Our case differs considerably from others in many respects: (i) in contrast to most urticaria-associated parasites, *D. dendriticum* is not a gastrointestinal parasite; (ii) in contrast to most parasite-associated urticaria, the presentation in our patient was acute, much like schistosome-caused urticaria; (iii) in contrast to schistosomal larvae, *D. dendriticum* does not require wandering in humans. Symptoms caused by *D. dendriticum* in rare cases of human infections include