PUBLICATIONS
Targeted detection of 65 kDa heat shock protein gene in endometrial biopsies for reliable diagnosis of genital tuberculosis

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ARTICLE INFO

Objective: To evaluate the clinical utility of PCR compared with other available diagnostic modalities in prompt diagnosis of female genital tuberculosis causing infertility. Study design: Prospective case-controlled trial. Premenstrual endometrial biopsy specimens were collected from 150 infertile women of reproductive age group suspected of having genital tuberculosis. All patients underwent diagnostic endoscopy (laparoscopy and hysteroscopy) and the samples obtained were subjected to microscopy, culture by the BACTEC 460 TB System, histopathology and polymerase chain reaction (PCR) for detection of 165 bp region of 65 kDa gene of Mycobacterium tuberculosis. The results were correlated with the laparoscopic findings. Results: While the laparoscopy/hysteroscopy findings were indicative of tuberculosis in 12.6% of cases, 14.6% of the specimens showed evidence of 65 kDa gene of M. tuberculosis and only 3.33%, 1.33% and 0.66% were positive by culture, smear and histopathology, respectively. Conclusion: Since laparoscopy, hysteroscopy other endoscopic procedures are associated with operative risks and may cause flaring of infection, and other conventional laboratory tests including histopathology have poor sensitivity, PCR-based detection of 65 kDa gene of M. tuberculosis in endometrial biopsy specimens could be a promising molecular diagnostic technique compared to conventional methods of diagnosis.

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1. Introduction

Mycobacterium tuberculosis (M. tuberculosis), the etiological agent for tuberculosis, has been extensively studied for over a century now. Despite the availability of anti-tubercular chemotherapy tuberculosis still remains a major health problem and is the leading cause of morbidity and mortality in many developing/resource-poor countries. Despite the efforts that are being made to control tuberculosis worldwide, countless numbers of people die with every passing year. Efforts in averting the disease have further been impeded by the synergistic relationship between tuberculosis and HIV, as tuberculosis, being an opportunistic infection worsens the immunological suppression in HIV patients. Early, accurate diagnosis and immediate curative treatment, under proper supervision to ensure that drugs are taken for the appropriate duration, is the key to disease control.

Among the various clinical presentations of tuberculosis, female genital tuberculosis poses serious concern throughout the world because of various associated complications like oligomenorrhea, amenorrhea, primary or secondary infertility, chronic pelvic pain, pelvic mass and significant mortality [1,3–7]. The most commonly affected organs are the fallopian tubes and endometrium, followed by the ovaries, cervix, vagina and vulva, and it is often associated with tubal blockage and pelvic, peritubal and perihilaradhesions (Fitz–Hugh–Curtis syndrome) [3,4,8,9]. Female genital tuberculosis commonly causes cessation and ulceration of the endometrium, resulting in destruction and partial or total obliteration of uterine cavity leading to Asherman’s syndrome [10,11]. In India, 5–16% is reportedly caused by tuberculosis, but the actual incidence rate may be underreported due to asymptomatic presentation of the disease and paucity of investigations.

Female genital tuberculosis caused by M. tuberculosis, presents invariably as a secondary complication acquired from an extragenital source like pulmonary or abdominal tuberculosis [3,4]. Effective management involves rapid, accurate diagnosis and early anti-tubercular treatment.
The diagnosis of tuberculosis still relies on acid fast bacilli (AFB) microscopy and culture, despite the fact that both techniques suffer several diagnostic lacunae, as reported earlier [1]. In samples derived from extrapulmonary sites that are often paucibacillary in nature, smear microscopy offers low sensitivity, requiring 10^9 bacilli/ml for detection. Culture, although being the gold standard, requires a minimum of 10–100 bacilli/ml and a long incubation period (by rapid test the earliest that mycobacteria can be detected is within twelve days) causing delay in diagnosis and treatment. Histopathological examination by haematoxylin and eosin for granulomatous tissue reactions compatible with tuberculosis infection is usually inconclusive, as reported earlier [2].

Over the last decade, polymerase chain reaction (PCR) has emerged as a rapid, sensitive and specific molecular method for detection of mycobacterial DNA by amplifying 65 kDa protein-encoding gene, 38 kDa antigen coding gene, the IS6110 and mpt64 gene in both pulmonary and extrapulmonary samples, as reported by various authors [4–6,8]. Subclinical disease or latent tuberculosis infection might give a positive result in PCR, but this is considered insignificant as prompt diagnosis is essential for averting permanent damage to genital organs and consequent infertility.

Endoscopic procedures like laparoscopy and hysteroscopy are widely used for investigation of infertile women [12–14]. Their role in the diagnosis of genital tuberculosis is well established. However, subclinical, latent or early stage infection may be overlooked during the procedure. A positive laboratory test may thus be helpful in timely diagnosis before extensive damage occurs.

Based on the aforesaid facts, the present prospective study was undertaken to assess the utility of PCR in definitive diagnosis of tuberculosis in Indian infertility patients in conjunction with endoscopic procedures – laparoscopy and hysteroscopy and conventional modalities (smear microscopy and culture based on the radiometric BACTEC system).

2. Materials and methods

The clinical part of the study was carried out at the IVF and Reproductive Biology Centre in the Department of Obstetrics and Gynecology, Maulana Azad Medical College, a teaching government hospital in Delhi, while the molecular typing was carried out at the National Centre for Disease Control (NCDC), Delhi. The institutional ethical committee approved the study.

2.1. Specimen collection

The study included 180 women between 18 and 40 years of age presenting to the infertility clinic for a period of 18 months from March ‘09. Additionally, 10 endometrial biopsy samples from patients with a diagnosis other than tuberculosis were collected and evaluated for the specificity of PCR in these samples. Women with a previous history of genital tuberculosis and male factor infertility were excluded from the study. A detailed history comprising menstrual, obstetrical and medical history, Mantoux status, erythrocyte sedimentation rate and previous history of tuberculosis was taken and a systemic abdominal and vaginal examination was performed. Consent was obtained from every patient.

2.2. Endometrial biopsy

Premenstrual endometrial biopsy was collected from all women attending the infertility clinic. Endometrial tissue obtained was collected in normal saline in a sterile container and immediately transported to the tuberculosis laboratory for further processing.

2.3. Microscopy and culture

The endometrial samples were processed as described by Chakravorty et al. [7]. The processed endometrial extract was microscopically examined for AFB, and 0.1 ml of the extract was inoculated into a BACTEC 12B vial along with antibiotics like PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) and benzyl penicillin.

2.4. DNA extraction

Bacterial DNA was extracted from 200 μl of the endometrial biopsy sample using the commercially available Qiagen DNA extraction kit (Qiagen, GmbH, 40724 Hilden, Germany) following the manufacturer’s protocol. An initial modification step was carried out by keeping the preliminary processed material at 80°C for 10 min for inactivation of the possible mycobacteria. The extracted DNA was stored at –20°C until use.

2.5. Polymerase chain reaction

PCR for the β-globin gene was carried out to ascertain the quality of the extracted DNA and verify that the isolated DNA is suitable for PCR amplification [15]. PCR was carried out to amplify the 165 bp region of the 65 kDa HSP gene of M. tuberculosis using previously reported primers [8]. The PCR was carried out in 25 μl volume consisting of the forward and reverse primers at final concentrations of 0.01 and 1 mM, respectively, 2.5 U of Taq polymerase (Perkin Elmer) in amplification buffer, 200 μM (each) of the four deoxyribonucleoside triphosphate and 5 μl of the extracted DNA [8,9]. Thermal profile included initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 63°C for 20 s, extension at 72°C for 1 min, and final extension at 72°C for 7 min. Each PCR was set up along with a positive control (H37Rv) and several negative controls (sterile distilled water) interspersed among samples to monitor cross-contamination. The amplified products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to check for the presence of specified bands.

2.6. Histopathology

Paraffin-embedded tissue sections were prepared and stained with haematoxylin–eosin and examined for granulomatous reactions suggestive of mycobacterial disease.

2.7. Laparoscopy/hysteroscopy

All patients underwent diagnostic endoscopy (laparoscopy and hysteroscopy) under general anesthesia post-menstrually using the three puncture technique. The laparohysteroscopic findings were subdivided into two types: suggestive which included affirmative findings (presence of tubercles, peritubal and/or periovarian adhesions, tubo-ovarian mass, beaded tubes, cornual blockage) and suspicious findings (hydrosalpinx, sacculated tubes, signs of chronic inflammation, pelvic inflammatory disease, and mild adhesions) while the second type were normal findings.

2.8. Statistical analysis

The population was divided into two groups of PCR positive and PCR negative women. Data analysis was performed using SPSS version 13 (SPSS Inc., Chicago, IL, USA). χ² test was used as test of independence. Value of P ≤ 0.05 was considered to be statistically significant.
3. Results

Of the 180 cases enrolled, 150 were included for the study: 18 women who did not undergo laparoscopy, 3 patients in whom culture could not be done and 9 patients whose PCR results were not reproducible or who did not undergo PCR testing were excluded from the study. According to Kuppuswami’s scale, the majority of the patients (73.2%) were found to be of poor socioeconomic status [16]. The mean age of the study population was 28.5 ± 4.6 years (range 18–40). Primary infertility was present in 108 women (72%) while 42 (28%) had secondary infertility. The duration of infertility ranged from 2 to 18 years.

A total of 22 (14.6%) women were diagnosed with genital tuberculosis on the basis of laboratory tests and laparoscopic/hysteroscopic findings. Among the samples, differences were observed between results for the detection of M. tuberculosis by AFB, histopathological evidence of tuberculosis infection, isolation by culture and detection of M. tuberculosis by PCR. PCR detected 22 (22/150; 14.6%), culture 5 (5/150; 0.033%) and AFB smear detected only 2 (2/150; 0.0133%) cases. All cases positive by AFB smear and culture were also found to be positive by PCR. M. tuberculosis isolates were identified by the standard biochemical criteria [17]. Granulomatous tissue reaction compatible with tuberculosis was observed only in 1 (1/150; 0.0067%) case.

Laparoscopic/hysteroscopic findings were pathognomonic for genital tuberculosis in 19 (12.67%) women while 131 (87.33%) women had normal findings. The various findings included adhesions; grade I (localized, covering one-third of the adnexa), grade II (moderate, covering one-third to two-thirds of the adnexa), caseation, endosalpingitis and obliterated pouch of Douglas (POD). Tubal factor etiology was most prevalent, with 90% afflicted patients out of whom 15% had a history of prior salpingectomy. The fallopian tube findings included cornual block, hydrosalpinx, pyosalpinx and beaded-tube appearance.

Various clinical parameters like the Mantoux test, ESR, previous history of tuberculosis and type of infertility were compared between the PCR positive and negative women. None of the tests showed a significant correlation between the PCR positive and PCR negative patients.

Among the PCR positive patients, past history of tuberculosis was present in 31.8% of patients in the form of pulmonary (42.8%), lymph node (28.5%) and bone tuberculosis (28.5%). Normal menstrual cycles were seen in 70.6% of cases while menstrual dysfunction in the form of amenorrhea, oligomenorrhea, menorrhagia and dysmenorrhea was seen in 8%, 13.33%, 7.3% and 0.67% of women, respectively. Oligomenorrhea was found to have a significant correlation between PCR positive and negative patients (P < 0.05). Endometriosis grade II (consistent with endometriosis, i.e. hemosiderin, characteristic glands and stroma) was a coincidental finding among 22.7% patients positive by PCR.

4. Comment

Female genital tuberculosis is a very common disease in developing countries which manifests itself as pelvic inflammatory disease in its acute form and later with menstrual irregularities and infertility, and is almost always secondary to a primary lesion elsewhere. The true incidence of the disease remains unknown as the disease poses diagnostic difficulties mainly because the primary symptoms are usually non-characteristic. Infertility is a well-known sequela. Early diagnosis invariably helps to speed up the decision-making process and markedly reduces the time lag in starting anti-tubercular therapy. Although the reported incidence of genital tuberculosis in Asian and western countries varies between 0.69% in Australia and 17.4% in India, the actual incidence may be higher because a large proportion of cases go unreported due to lack of sensitive and specific investigations [18,19]. Factors such as poverty, homelessness, a poorly functioning national tuberculosis program and dismantling of public health infrastructure have significantly contributed to the worsening situation.

To qualify as a diagnostic test for genital tuberculosis, a combination of early diagnosis along with good sensitivity and specificity is required. In the present study, we evaluated the acceptability of PCR as a diagnostic test for female genital tuberculosis and also correlated the findings of PCR with laparoscopy/hysteroscopy and other diagnostic tests. A good correlation between PCR and laparoscopy/hysteroscopy results was found. The main advantage of PCR is that it is a rapid and specific molecular technique which allows detection of mycobacteria from both pulmonary and extra-pulmonary specimens within 4–5 h, compared to culture which has a poor detection rate and requires a minimum of 12 days to get the result [11,20,21]. Histopathology was inconclusive in a maximum number of cases. This is in agreement with an earlier study by Kumar et al. [2]. In this prospective study, we found that PCR alone could detect the presence of M. tuberculosis DNA in 14.6% cases while AFB culture and AFB smear showed a poor detection rate of 0.033% and 0.013% respectively, thus suggesting an appreciable and sensitive nature of PCR in detection of mycobacteria.

In our study, PCR was positive in all the cases with affirmative or suspicious findings on endoscopy while detecting an additional 5 cases which showed normal laparoscopic findings. This could be attributed to the fact that while laparoscopy/hysteroscopy detects conspicuous changes such as pelvic/intrauterine adhesions, tubercles, grade I endometriosis with the tubes being the commonest location, subtle changes might be overlooked. Thus an additional 3 women diagnosed with genital tuberculosis by PCR may be considered as harboring a sub-clinical or latent infection, the early diagnosis of which could have a significant role in regaining fertility.

Improvement in pregnancy rates after tuberculosis treatment has been reported earlier [23,24]. It is thus imperative to diagnose the disease at an early stage, thus preventing tubal damage and subsequent infertility. Endoscopic procedures like laparoscopy and hysteroscopy, although considered as the gold standard for diagnosing genital tuberculosis, have associated risks of increased complication rates during the procedure and postoperative flare-up of insidious disease though no operative risk and flaring of infection was seen in our study [21,25]. High complication rates associated with endoscopic procedures support the use of PCR in diagnosis of tuberculosis.

Stringent quality control measures were adopted while carrying out PCR. Several negative controls were interspersed between samples to avoid cross-contamination thus preventing false positive results.
Menstrual dysfunction complaints were seen in 29.3% of patients. Oligomenorrhea was found to have a significant correlation between PCR positive and negative women, indicating that tuberculosis bacilli cause scarring of the endometrial lining. Partial or total destruction of endometrium by the disease process resulting amenorrhea has been reported in a few cases.

The gross appearance of endometrium was mostly unremarkable. In advanced cases, however, ulcerative or atrophic endometrium and an obliterated endometrial cavity due to extensive intrauterine adhesions was seen on endoscopy. Endometriosis grade II was observed in 22.7% of PCR positive patients. This can be attributed to the fact that endometriosis, being an auto-immune disease, has been shown to create a defect in natural killer activity resulting in decreased cytotoxicity to autologous endometrium. This immune defect may account for the incidence of other infections including tuberculosis occurring more frequently in these women [22].

The prevalence of female genital tuberculosis in infertility clinics has been reported to range from 1 to 19% [4,26–28]. Atypical clinical presentation and failure of the laboratory tests currently used have probably entailed significant underreporting of the actual prevalence of genital tuberculosis.

Our results show that PCR-based detection of M. tuberculosis in endometrial biopsy specimens is a sensitive technique for pre-emptive vigilance of possible reactivation for genital tuberculosis, which is a leading cause of infertility in developing countries. In the absence of a gold standard, PCR is being suggested as a gold standard diagnostic modality for diagnosis of genital tuberculosis in view of its high sensitivity and specificity. Moreover, since performing laparoscopy and other endoscopic procedures on every patient is not feasible in practice, PCR qualifies as a promising molecular diagnostic technique in the near future.

Conflict of interest

None.

Acknowledgements

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LETTER TO THE EDITOR—BRIEF COMMUNICATION

Rare case of multi-drug resistant endometrial tuberculosis unveiled by DNA signature studies of the rpoB, katG and inhA genes

Dear Editor,

We have found a case of multi-drug resistant (MDR) endometrial tuberculosis unveiled by DNA signature studies of the rpoB, katG and inhA genes.

A 29-year-old woman presented to our clinic with primary infertility for seven years and oligomenorrhea for one year. She reported no weight loss, fatigue, lack of appetite, elevation of temperature or other systemic symptoms. She revealed a history of skin tuberculosis two years previously on the basis of excision biopsy. General physical and pelvic examination was normal. The chest radiograph and routine biochemical investigations including ESR and Mantoux test were normal. The investigations for infertility such as D–A2 hormonal assay and semen analysis were normal. Pelvic ultrasound showed endometrial thickness of maximum 4.5 mm with hypeechoic shadows in the endometrial cavity at the fundus signifying sub-endometrial calcification thus raising the suspicion of genital tuberculosis (Fig. 1). Laparoscopy revealed no free fluid, though bilateral terminal hydrosalpinx, no free peritoneal spill and adhesions in the perihepatic region and Pouch of Douglas (POD) were found. Peritoneal and endometrial biopsy samples for AFB smear/culture on BACTEC 460 TB system and histopathology were negative for Mycobacterium tuberculosis.

In recent years, DNA PCR for detection of M. tuberculosis-specific genes (primarily 65 kDa protein-encoding gene, along with other targets like 38 kDa antigen coding gene, IS6110 and mpt64 gene), has offered great promise in early and rapid diagnosis of both pulmonary and extrapulmonary tuberculosis, including our recent report for laboratory diagnosis of female genital tuberculosis using DNA PCR for 65 kDa heat shock protein gene of M. tuberculosis [1–5]. In our study, the patient tested positive for 165 bp region of 65 kDa gene-based DNA PCR. Subsequently, the sample was subjected to automated DNA sequencing for 350 bp region of rpoB gene, 620 bp region of katG gene and 248 bp region of inhA genes of M. tuberculosis using the Big Dye-terminator cycle sequencing kit (ABI, USA) using rpoB, katG and inhA gene-specific primers [6,7].

Genomic data analysis revealed a D516Y (Gene bank accession number JF268609) mutation in the rpoB gene and S315T mutation in the katG gene.

The patient was followed up for three months: (i) initially when she was diagnosed as a case of MDR genital tuberculosis based on our PCR result, she was immediately put on second line anti-tubercular drug regimen as per the revised tuberculosis control programme for multidrug-resistant tuberculosis; (ii) she was again followed up after a gap of three months following which her endometrial thickness improved significantly from ~4.5 mm to 7 mm. No other significant clinical signs and symptoms were noted. Subsequently, she was counselled for undergoing Assisted Reproductive Technology (ART) but she and her spouse informed us that they were not willing for this due to financial reasons.

Female genital tuberculosis is being increasingly recognised as a notable cause of infertility in recent years. Prevalence of the disease varies widely from 0.69% in Australia to 19% in India in women of reproductive age group, mostly as a secondary complication to the primary focus elsewhere in the body [8]. In view of the ‘dormant’ clinical presentation, the diagnosis of this disease requires a multi-disciplinary approach involving clinical examination, endoscopic laparoscopy and laboratory findings. Since laparoscopy detects only macroscopic changes which are manifested during later stages of the disease; and AFB culture is often negative in such cases, current molecular techniques may help in early diagnosis [8]. In addition, sequence-based monitoring of mutation(s) in the defined targets of drug-resistance genes may also improve the treatment decision-making process.

The clinical diagnosis of the patient of this study was suggested of underlying endometrial tuberculosis but AFB smear and culture were negative. Strong clinical suspicion, and history of skin tuberculosis only two years previously, prompted us to go for diagnostic PCR which showed definitive evidence of M. tuberculosis-specific 65 kDa gene, confirming the diagnosis of the case as endometrial tuberculosis. Interestingly, sequence analysis showed classical drug-resistance mutations at codon 516 in rpoB gene and at codon 315 in the katG gene. Although, multi-drug resistant tuberculosis of lung and some other anatomical sites is common, we have not come across any report of multi-drug resistant tuberculosis of the endometrium, at least from India.

Fig. 1. Transvaginal sonography showing sub-endometrial calcification.
It may therefore be worthwhile to rule out the possibility of culture-negative subclinical cases of genital/endometrial tuberculosis in infertile patients with suspicion of pelvic inflammatory disease by applying nucleic acid amplification-based techniques, thus preventing irreversible damage to the genital organs.

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References


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Genetic analysis of precore/core and partial pol genes in an unprecedented outbreak of fulminant hepatitis B in India


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SHORT REPORT

Genetic analysis of precore/core and partial pol genes in an unprecedented outbreak of fulminant hepatitis B in India

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SUMMARY

We investigated an unprecedented outbreak of fulminant hepatitis B virus (HBV) that occurred in Modasa, Gujarat (India) in 2009. Genomic analysis of all fulminant hepatic failure cases confirmed exclusive predominance of subgenotype D1. A1762T, G1764A basal core promoter (BCP) mutations, insertion of isoleucine after nt 1843, stop codon mutation G1896A, G1862T transversion plus seven other mutations in the core gene caused inhibition of HBeAg expression implicating them as circulating precore/BCP mutant virus. Two rare mutations at amino acids 89 (Ile→Ala) and 119 (Leu→Ser) in addition to other mutations in the polymerase (pol) gene may have caused some alteration in either of four pol gene domains to affect encapsidation of pregenomic RNA to enhance pathogenicity. Sequence similarity among patients’ sequences suggested an involvement of a single hepatitis B mutant strain/source to corroborate the finding of gross and continued usage of HBV mutant-contaminated syringes/needles by a physician which resulted in this unprecedented outbreak of fulminant hepatitis B. The fulminant exacerbation of the disease might be attributed to mutations in the BCP/precore/core and pol genes that may have occurred due to selection pressure during rapid spread/mutation of the virus.

Key words: HBV, polymerase (pol) gene, precore/core gene.

Hepatitis B virus (HBV) infections lead to a wide spectrum of liver injuries ranging from acute self-limiting infection and fulminant hepatitis to chronic hepatitis, cirrhosis and hepatocarcinoma. Although, HBV infection is predominantly of sporadic nature, epidemics in the past have been reported mainly in healthcare settings highlighting the failure of healthcare personnel to adhere to good medical practice and infection control measures. The USA reported 33 outbreaks of HBV or hepatitis C virus (HCV) from 1998 to 2008 [1]. In India, an outbreak of HBV with high mortality (7/15, 46.7%) based on serological diagnosis confirming anti-delta positivity in three cases including one survivor with no molecular data was reported in Gujarat in 1997 [2]. Around February–March 2009, one of the most severe outbreaks of HBV infection erupted suddenly around the Modasa township of Gujarat state in India, affecting over 500 people and causing the death
of 93 people within just 3–4 weeks [3, 4]. The characteristic of this outbreak was its short incubation period; all patients tested positive for HBsAg with high serum levels of alanine aminotransferase (ALT) and rapid development of fulminant hepatitis leading to death from hepatic and renal failure. Epidemiological investigation traced the source of infection to contaminated syringes and needles, as all dying patients revealed a history of having received therapeutic injections from the same physician, which led to his arrest and the closure of his clinic. Violation of infection control practices became a major news story after the outbreak. Considering the aforesaid details, we investigated this outbreak for the possible link of specific mutations in the basal core promoter (BCP)/precore/core and partial pol genes of the circulating HBV strains with fulminant exacerbation of the disease outbreak.

Since the National Centre for Disease Control (NCDC) was officially designated as the investigating agency, the HBV outbreak investigation did not require any prior ethical clearance. The present study was designed based on the case definition of an acute case according to national guidelines, i.e. the patient should be positive for hepatitis B surface antigen (HBsAg), and have an ALT level of >400 IU. Serum samples from 21 acute fulminant hepatic failure (FHF) cases and 10 acute, self-limiting hepatitis B (ASH) cases were collected and transported to NCDC under cold conditions (i.e. cold packs maintained at 0–4 °C). Although the sample size was not adequate, probably due to the high number of deaths causing health officials to focus mainly on curbing mortality, which limited the number of samples they collected. However, with complete outbreak details available, we were able to closely observe the demographic details (Table 1). All 21 FHF patients had a history of receiving injections during the last 6 months at the clinic of the suspected physician. All the serum samples were tested for HBsAg, antibodies to hepatitis B surface antigen (anti-HBs), hepatitis B e antigen (HBeAg), antibody to HBeAg (anti-HBe), anti-HBc (immunoglobulin IgG and IgM), anti-HAV (IgG and IgM), anti-HCV and anti-HEV by microparticle enzyme immunoassay (Abbott Laboratories, USA) according to the manufacturer’s protocol at the central Hepatitis Laboratory of our institute. Viral DNA was extracted from serum samples using a commercial DNA mini kit (Qiagen GmbH, Germany). A nucleotide (nt) sequence of around 1078 bp encoding for precore/core and partial pol genes was amplified with forward primer (5’-GGAGTTGCGAGGAGG-3’) (nt 1736–1755) and reverse primer (5’-AGGGCTACGTCTTCTTCT-3’) (nt 2786–2805) designed with Primer 3 (v. 0.4.0) software (http://fokker.wi.mit.edu/primer3/). Next, 5 μl DNA was used for PCR in a total reaction volume of 25 μl containing 10 pmol of each oligonucleotide in a 1 x PCR reaction buffer (final concentration): 2.5 mM MgCl₂, 200 μM dATP, dGTP, dCTP, dTTP and 2.5 U AmpliTaq Gold (Applied Biosystems, USA). The thermal cycling parameters included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The amplified product was purified and sequenced from both directions using fluorescence-labelled dideoxynucleotides on an automated DNA sequencer (ABI 310 Genetic Analyser; Applied Biosystems, USA) using the dideoxy terminator cycle sequencing chemistry following the protocol used by us previously [5]. For sequence alignment and phylogenetic analysis, we selected the GenBank sequences with the best and highest scoring matches with our sequences in a NCBI BLAST search. Multiple sequence alignment was performed using Bioedit version 7.0.4.1 [6]. Genetic distances were calculated using the Kimura two-parameter algorithm and a phylogenetic tree was constructed by the neighbour-joining method. To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were performed 1000 times. Phylogenetic analysis was done using MEGA version 4.1 [7]. The unpaired t test was used to compare group means of nucleotide sequence divergences. Two-tailed P values of <0.05 were considered statistically significant. Calculations were performed using Stata statistical software (release 5.0; Stata Corporation, USA). The χ² test was used to compare different

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Table 1. Number and percentage of cases and deaths for age group and gender
attributes in demographic analysis (Table 1). The new sequences obtained in this study were submitted to EMBL and assigned accession numbers JQ287601–JQ287621.

Demographic analysis revealed a total of 560 hepatitis B cases reported with 93 (16.60%) deaths in one of the most lethal outbreaks ever reported in India. Statistically significant difference was observed between 61.07% male and 38.92% female cases reported in this hepatitis B outbreak \((P < 0.05)\) (Table 1). The maximum number of cases, i.e. 429 (76.60%) cases with 65 (15.15%) deaths, were reported in the 13–45 years age group, followed by 102 (18.20%) cases with 25 (24.50%) deaths in the >45 years age group, the least number of cases occurred in the 0–12 years age group (29 cases, 5.17%; three deaths, 10.30%). Considering the percentage of deaths in each age group, we observed no significant difference between different age groups \((P > 0.05)\). Serological analysis revealed that all 21 FHF samples tested positive for HBsAg and HBe IgM suggesting that all the cases were of acute HBV infection further confirming the aetiology of the outbreak. HBeAg was detected in six cases and found negative in 15 cases. Anti-HBe was detected in 12 cases and both markers were detected in three cases. None of the samples were reported to be positive for HDV or other hepatitis markers (HAV, HCV, HEV), thereby excluding any possibility of co-infection with other hepatitis viruses. Moreover, the level of ALT, an indicator of liver necrosis, was determined. Compared to normal levels of 7-56 IU/l, the average serum ALT level found in the fulminant cases was 2391 IU/l. The serum ALT levels exhibited a marked 46-fold rise, with reference to the upper limit of the normal range. Of the 10 ASH samples, all were found positive for HBsAg and anti-HBc IgM; nine were further positive for HBeAg and one positive for anti-HBe.

Sequencing analysis revealed 19 missense mutations exclusively in the 21 FHF samples and none in the 10 ASH serum samples: two in the BCP region (A1762T, G1764A), 10 in the C region (three in pre-core and seven in core) which notably included an isoleucine (Ile, ATC) insertion after nt 1843, G1862A and G1896A transversion, and seven in the partial P region, among which two rare mutations were

<table>
<thead>
<tr>
<th>Region</th>
<th>Amino acid*</th>
<th>Nucleotide (nt position)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP (nt 1742–1849)</td>
<td>7</td>
<td>1762</td>
<td>AAA (Lys)→AAT (Asn)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1764</td>
<td>GGT (Gly)→GAT (Asp)</td>
</tr>
<tr>
<td>Precore (nt 1814–1897)</td>
<td>11</td>
<td>1843</td>
<td>Insertion of Ile (ATC)</td>
</tr>
<tr>
<td></td>
<td>17†</td>
<td>1862</td>
<td>GTT (Val)→TTT (Phe)</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1896</td>
<td>TGG (Trp)→TAG (stop codon)</td>
</tr>
<tr>
<td>Core (nt 1901–2452)</td>
<td>56</td>
<td>1979, 1981</td>
<td>ATT (Ile)→GTA (Val)</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>2020</td>
<td>GAG (Glu)→GAT (Asp)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>2100</td>
<td>AAT (Asn)→ACT (Thr)</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>2121</td>
<td>AGT (Ser)→AAT (Asn)</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>2149</td>
<td>GAA (glu)→GAT (Asp)</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>2160, 2161</td>
<td>AGC (Ser)→AAT (Asn)</td>
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<tr>
<td></td>
<td>120</td>
<td>2171, 2172</td>
<td>GTT (Val)→ACT (Thr)</td>
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<tr>
<td>Reverse transcriptase/pol</td>
<td>69</td>
<td>2511</td>
<td>GTC (Val)→ATC (Ile)</td>
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<tr>
<td>(nt 2307–3182, 1–1623)</td>
<td>73</td>
<td>2525</td>
<td>GAT (Asp)→GAA (Glu)</td>
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<tr>
<td></td>
<td>75</td>
<td>2529</td>
<td>AAA (Lys)→CAA (Gln)</td>
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<td></td>
<td>81</td>
<td>2547, 2549</td>
<td>CAC (His)→AAA (Lys)</td>
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<tr>
<td></td>
<td>85</td>
<td>2561</td>
<td>CAG (Gln)→CAT (His)</td>
</tr>
<tr>
<td></td>
<td>89†</td>
<td>2571, 2572</td>
<td>ATT (Ile)→GCT (Ala)</td>
</tr>
<tr>
<td></td>
<td>119‡</td>
<td>2661, 2662, 2663</td>
<td>CTT (Leu)→AGC (Ser)</td>
</tr>
</tbody>
</table>

* Amino acid positions are numbered from the start codon of each protein.
† Four strain HM042274, HM042275, HM042279 and HM042280 were observed with this mutation.
‡ Rare mutation.
observed at amino-acid positions 89 and 119 (Table 2). Phylogenetic analysis of our sequences with database sequences revealed that all 21 FHF cases belonged to genotype D (subgenotype) (Fig. 1). Analysis of the 10 ASH cases revealed their similarity to wild-type precore/core and polymerase (pol) gene sequences which ruled out the possibility that HBV strains responsible for this outbreak pre-existed in this geographical area. Among these cases eight were found to belong to genotype D and two to genotype A (data not shown).

High mortality during fulminant hepatitis B outbreaks poses a serious threat to a vulnerable community. Since studies on fulminant hepatitis B are usually performed late in the course of the disease, it was difficult to reconstruct the sequence of virological and immunological events that led to mass hepatocellular injury [8]. In terms of the percentage acquisition of infection in males and females, a predilection of acquisition in males was observed. This could be attributed to the fact that in Gujarat, a state with poor literacy and a skewed sex ratio, females may not take good care of their own health, resulting in a very small percentage of women attending clinics for their illness. Thus, fewer females were affected in this deadly HBV outbreak which was epidemiologically linked with repeated usage of syringes and needles. In terms of the percentage of deaths, numbers were equally distributed among the sexes. Thus, the female physiology did not affect the severity of the infection. This outbreak was significant for its rapid spread and high mortality which encouraged us to discover the possible cause and source of the outbreak. Although unusual, the outbreak was evidenced by the presence of HBsAg and IgM anti-HBc in the patients. Considering co-infection with HDV as a possible reason for high mortality, all the cases were screened for HDV and other hepatitis viruses such as HAV, HEC or HCV. None of the samples were found positive for co-infection which excluded this as the possible reason for the outbreak. Epidemiological investigations by health officials identified an association of cases with a history of injection and a physician treating very large numbers of patients on the basis that all 40 dying patients gave information about receiving injections from the suspected physician. This physician was later arrested and his clinic closed down following a local court directive. The need for stringent legal action was seen as necessary to deal with such a case of medical negligence which would otherwise have caused further fatalities in the population.

This outbreak prompted healthcare authorities to drive the campaign to check for the compliance of local hospitals regarding their infection control practices. Similarly, a study by Samandari et al. found a lack of infection control measures in the USA where a large outbreak of HBV infection was associated with frequent injections from a physician [9]; while Harpaz et al. implicated a surgeon in the transmission of HBV to 19 patients [10]. Phylogenetic analysis of genotype D1 and close sequence homology in all FHF cases strongly indicate the involvement of a single hepatitis B mutant strain/source. Investigating the same outbreak, Arankalle et al. reported the presence of precore and BCP mutants and four amino acid substitutions, genotype D with D1 being present in all FHF cases while D2 was associated with self-limiting acute viral hepatitis cases [4]. Expanding on the findings of Arankalle et al. we surmise that BCP mutations at nt 1762 and nt 1764, insertion of Ile after nt 1843, transversion of G to T at nt 1862 (changing the specificity of codon 17 from valine to phenylalanine, responsible for abrogating cleavage of p25 by the cellular signal peptidase), and transition of G to A at nt 1896 led to the creation of a premature stop codon in the ORF of the precore region by truncating the precore/core protein into a 28-amino-acid peptide against the original 29-amino-acid precore and 181-amino-acid core genes. This resulted in inhibition of HBeAg expression through transcriptional downregulation and enhanced viral replication which implicated all 21 cases to be circulating BCP/precore mutants; and the probable association of these mutant viruses with fulminant hepatitis and mortality, as HBeAg is a known immunomodulator that prevents exceedingly strong cytotoxic immune responses, as suggested in previous studies [4, 11–13]. This is further supported by serological analysis where the presence of HBeAg in six patients, anti-HBe in 12 and both markers in three patients indicated rapid mutation of the virus to convert into the mutant form which probably took over the initially circulating wild virus. By contrast, none of the reported mutations were found in the self-limiting HBV cases whose sequences exhibited a wild-type pattern. Further, the outbreak isolates were found to exhibit mutations of unknown clinical significance in the precore/core and partial pol genes, which may predict these HBV isolates as mutants divergent from all known DNA sequences; however, the significance of these mutations in the pathogenesis of the fulminant exacerbation of the disease needs to be elucidated [14]. All observed
Fig. 1. Phylogenetic analysis constructed using MEGA software version 4.0.2 showing our sequences, with representative sequences of all HBV genotypes identified by respective accession number and genotype, along with bootstrap values.
Thakur et al. found that genotype D was associated with severe liver disease compared to genotype A [20]. We conclude with the view that the BCP/precore mutant, the mutation in core and partial pol genes along with the finding of genotype D (subgenotype D1), and the clinical presentation of cases in terms of positive HBsAg, elevated ALT indicative of hepatocyte damage, liver necrosis, and enhanced level of anti-HBc cited in the study could corroborate the statement that this outbreak was caused by a mutant HBV strain having high virulence and replication. The finding of this study strongly warrants periodical monitoring of the discharged treated cases to check for virus clearance in order to curb its further spread and strenuous efforts to increase awareness of blood-borne infection among medical practitioners, and the public at risk, to ensure that standard precautions are always practised, and also to restrict the re-use of syringes and needles to prevent this often fatal infection. We believe all medical practitioners found guilty of medical negligence leading to the loss of human life must be deprived of their medical degree with an accompanying legal punishment in order to curb such serious human error.

ACKNOWLEDGEMENTS

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DECLARATION OF INTEREST

None.

REFERENCES


