Prospective evaluation of the threat related to the use of seminal fractions from hepatitis C virus-infected men in assisted reproductive techniques

T. Bourlet1, J. Lornage2, A. Maertens1, A.-S. Garret2, H. Saoudin1, J.-C. Tardy3, C. Jimenez4, J.-F. Guerin2, B. Pozzetto1,6, and R. Levy1,5

1Laboratoire de Bactériologie-Virologie, GIMAP EA 3064, IFRESIS, Faculté de Médecine J. Lisfranc, Université de Saint-Etienne, 15 rue Ambroise Paré, 42023 Saint-Etienne Cedex 02, France
2Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire de Lyon, Lyon, France
3Laboratoire de Virologie, Centre Hospitalier Universitaire de Lyon, Lyon, France
4Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire de Dijon, Dijon, France
5Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire de Saint-Etienne, Saint-Etienne, France
6Correspondence address. Tel: +33-4-77-82-83-15; Fax: +33-4-77-82-84-60; E-mail: bruno.pozzetto@univ-st-etienne.fr

BACKGROUND: The risk of hepatitis C virus (HCV) transmission during assisted reproductive techniques (ARTs) is still disputed and no report concerning its prospective evaluation is available.

METHODS: The aim of this 4-year follow-up multicentre study that enrolled 86 HCV-serodiscordant couples was to determine whether a sperm-processing method was able to reduce levels of HCV in semen and the risk of HCV transmission to the newborn. All the men were chronically infected by HCV and 10 of them by human immunodeficiency virus. A total of 181 seminal plasmas and 153 sperm fractions were tested for the presence of HCV RNA.

RESULTS: HCV RNA tested positive in 20.4% of the seminal samples. All of the 153 final sperm fractions tested negative for HCV. The detection of HCV RNA in semen was significantly correlated with a high viral load in blood (P < 0.05). The presence of HCV RNA in seminal plasma impaired neither semen parameters nor ART issue. From the 58 couples enrolled effectively in an ART programme, 24 pregnancies and 28 newborns were obtained. All of them tested negative for HCV RNA in blood.

CONCLUSION: These results emphasize the safety of the semen-processing method. The negligible risk of transmitting HCV reduces the value of the systematic analysis of HCV RNA in seminal fractions prior to ART. Since use of this analytical procedure involves the freezing of semen, its avoidance would result in an increase in sperm quality and reduce the need to perform intracytoplasmic sperm injection techniques.

Key words: hepatitis C virus / semen / assisted reproduction techniques / viral risk / human immunodeficiency virus

Introduction

The sensitivity of hepatitis C virus (HCV) RNA detection in seminal plasma has been improved by using conventional or real-time PCR assays (Leruez-Ville et al., 2000; Bourlet et al., 2002; Bourlet et al., 2003; Halfon et al., 2006; Pasquier et al., 2006). Using these techniques, the authors reported a prevalence of seminal HCV RNA in chronically HCV-infected men, varying from 15 to >30% in patients co-infected by HCV and human immunodeficiency virus (HIV) (Leruez-Ville et al., 2000; Levy et al., 2000; Bourlet et al., 2002; Nyamathi et al., 2002; Pasquier et al., 2003; Pekler et al., 2003). Despite the fact that about a quarter of seminal plasma samples from HCV chronically infected men contain HCV RNA, the sexual transmission of HCV remains a rare event (Neumayr et al., 1999; Zylberberg et al., 1999); when the man is co-infected by HIV, the risk of transmitting HCV by sexual intercourse is slightly increased (Wyld et al., 1997).

The risk of HCV transmission during assisted reproductive technique (ART) procedures is not documented and no report concerning the prospective evaluation of ART in terms of HCV transmission is available. HCV RNA has not been reported to be present in the sperm fractions used for ART (Mckee et al., 1996; Garrido et al., 2005) with the exception of one observation (Bourlet et al., 2002). In the latter case report, the sperm fraction selected after a washing procedure was found positive for HCV RNA detection; reassuringly, this fraction was found negative when the...
density-gradient centrifugation was followed by an additional ‘swim-up’ step. As a precaution, specific guidelines were introduced in France for the management of couples where the male partner was chronically infected by HCV (Anonymous, 1999). In addition to the use of a dedicated laboratory for the preparation of semen fractions, these recommendations include an analytical procedure using RT–PCR for the detection of HCV RNA in seminal plasma and, if positive, in the cellular purified fraction. However, this additional freezing/thawing step may impair the survival of spermatozoa and consequently the success of ART attempts.

The present study was conducted to assess prospectively the viral risk in HCV-serodiscordant couples entering an ART programme. The aims were to evaluate (i) the prevalence of HCV RNA in seminal plasma and sperm fractions used for ART, (ii) the influence of the presence of seminal HCV RNA on semen parameters and on ART outcome and (iii) the serological status of the babies conceived by ART in this context.

Materials and Methods

Patients

Eighty-six couples were included in a multicentre prospective study (sponsored by the University Hospital of Saint-Etienne) between July 2001 and December 2005, after they gave their written informed consent (CCPPRB Rhône-Alpes).

The infertility centres from three French university hospitals (Lyon, Dijon, Saint-Etienne) were included in the study. Seventy-six men were chronically infected by HCV and a further 10 were co-infected by HIV and HCV. All the women tested negative for both viruses. The mean age was 39.4 (range: 29–59) and 35.1 (range: 22–43) years for men and women, respectively.

Methods

Samples

A total of 148 blood samples, 181 seminal plasmas and 153 sperm cell fractions were collected. Plasma samples were separated from blood by centrifugation and frozen at −80°C until use.

Sperm treatment

Semen samples were obtained by masturbation into a sterile container after 3 days of sexual abstinence and processed within 2 h of ejaculation. One millilitre of the semen sample was centrifuged at 800 g for 10 min and the seminal plasma was separated from the cell pellet and stored at −80°C until further use. Another millilitre was submitted to centrifugation through a three-layer (3 ml of 50, 70 and 90%, respectively) discontinuous Pure-Sperm® gradient (Nidacon, Sweden) and washed in BM1 (Ellios Biotek, France); the motile spermatozoa were recovered from the 90% fraction, numbered under microscopic examination and separated into two aliquots, one kept frozen at −80°C for further virological analysis and another in liquid nitrogen for ART. When the number of spermatozoa was at least of 5 millions per millilitre, a swim-up test was performed by layering 0.5 ml of BM1 on the top of the 90% fraction obtained after Pure-Sperm selection; after incubation for 45 min at 37°C in a 5% CO2 incubator, the upper 0.5 ml fraction was carefully removed and the spermatozoa numbered. This swim-up fraction was kept frozen at −80°C until use.

Virological assays

The quantification of HCV RNA in blood plasma was achieved using the Cobas Amplicor HCV Monitor assay 2.0 (Roche Diagnostics, France) for 134 samples and the RealTime HCVTM assay (Abbott Molecular System, France) for 14 samples, according to the manufacturers’ instructions.

The presence of HCV RNA in seminal plasma was estimated using the same tests (163 samples with the Roche assay and 18 samples with the Abbott assay) but adapted extraction protocols, as described previously (Bourlet et al., 2002; Bourlet et al., 2006). Briefly, the extraction of RNA was performed by combining a lysis step with proteinase K (50 mg/ml) with the use of the RNAeasy Mini Kit (Qiagen, France). With reference to the standard Qiagen protocol, an additional elution step of the column was performed with the first eluate. The internal control of the Cobas Amplicor® HCV kit was diluted 1:16 in 50 μl of the column eluate. RT and qualitative PCR were performed according to the manufacturer’s instructions. The threshold of the qualitative detection of HCV RNA using the Cobas Amplicor HCV kit was estimated to be approximately 40 RNA copies/ml in seminal plasma samples (Bourlet et al., 2006). The determination of the viral load of the seminal plasma samples found positive by qualitative test was performed by using the RealTime HCV assay (Abbott Molecular System, France) (threshold of 12 IU/ml).

The presence of HCV RNA in sperm fractions was estimated as described previously (Bourlet et al., 2002). In addition, an extraction control was performed by RT–PCR amplification of an intron-containing region of the protamin 2 gene, as described previously (Miller et al., 1994). The threshold of 500 000 cells in PCR experiments was justified by the reproductive amplification of the control gene at this cell concentration but not at a lower one.

When a pregnancy occurred, the newborn was tested within 3 months after birth, for the presence of anti-HCV and anti-HIV antibodies.

Semen analysis and ART

Semen parameters were evaluated according to the criteria of the World Health Organization (1999). In vitro fertilization (IVF), frozen embryo transfer, intracytoplasmic sperm injection (ICSI) and intrauterine insemination (IUI) were offered to couples according to the French guidelines (Anonymous, 1999).

Statistical analysis

Data were analysed using Epi-info (6.04 CD) and SPSS (7.5, Chicago, USA) software. Qualitative variables were compared by Fisher’s exact test. For continuous variables, mean comparisons were based on Student’s t-test. All the statistical tests were interpreted at the 5% significance level.

Results

Characteristics of the studied population

Risk factors for HCV contamination were intravenous drug usage in 36 cases (41.8%), blood transfusion in 15 cases (17.4%), tattooing or piercing in 4 cases (4.7%) and undetermined in 31 cases (36.1%).

The repartition of HCV genotypes was as follows: 1b (32.7%), 1a (26.2%), 2a (16.4%), 3a (14.7%), 4 (3.3%), 2a (1.7%) and non-typeable (5%). Forty-one patients were tested for transaminases: the mean values were 76.5 (±73.4) and 51.8 (±49.1) IU/ml for aspartate transaminase and alanine transaminase, respectively. The histological analysis performed on hepatic biopsies in 27 men exhibited a moderate Metavir score (≤A1/F1) in most cases. Infertility was of female, male and mixed origin in 25, 13 and 62% of the cases, respectively. The main cause of male infertility was abnormal semen parameters (61%); tubarian (31%) and uterine lesions (18%) were the main causes of female infertility.

Virological data in HCV-infected men

Seminal samples tested positive for HCV RNA in 20.4% of cases (37 from 181) (20.2 and 22.2% in men infected by HCV and HIV/HCV, respectively).
When all the samples were taken into consideration, including multiple specimens from individual men, the mean HCV RNA load in blood was 5.84 ± 0.76 log IU/ml. No statistically significant difference was noted between the mean viral load of patients infected by HCV alone and that of patients co-infected by HIV and HCV (5.83 ± 0.75 versus 5.89 ± 0.87 log IU/ml).

The mean blood viral load was higher in subjects whose semen was found to be positive for HCV RNA than in those found negative (6.08 ± 0.58 versus 5.77 ± 0.88 log copies/ml, P < 0.05 by Student’s test). As shown in Table I, 22 men (26.2%) produced at least one positive seminal plasma sample during the study. The prevalence of samples found to be positive for HCV RNA was significantly higher when several sequential specimens were collected (P < 0.02 in Table I). From the 22 subjects found positive in at least one SP specimen by the qualitative method, HCV RNA was quantified in 21 in a positive sample of seminal plasma kept frozen at −80°C. From these 21 samples, 4 were found to be below the threshold of 12 IU/ml; the median viral load of the 17 remaining samples was 515 IU/ml (range: 14–6470). The mean difference value of viral loads obtained in blood and seminal plasma was 3.86 ± 0.78 log UI/ml. The correlation rate between these two series of viral loads was 0.42 (P < 0.05).

All the 153 final sperm fractions were found to be negative for HCV RNA in contrast to the housekeeping gene that was detected in all cases.

ART results

As shown in Table II, the presence of HCV RNA in seminal plasma did not impair the semen parameters of the men involved in an ART programme. Although there was a slight trend for an increase in the number of leucocytes in seminal plasma in the men whose seminal plasma tested positive for HCV RNA, this was not statistically significant (P = 0.06). The ART parameters evaluated for IVF and ICSI, including the number of top-quality embryos (Van Royen et al., 1999), the fertilization rate, the number of clinical pregnancies and the number of take-home babies per aspiration, were equivalent regardless of whether the seminal plasma was found to be positive or negative for HCV RNA (Table II).

During the study, a total of 135 fertilization cycles was completed, leading to 36 pregnancies, 12 spontaneous miscarriages and 28 births including four twins and one perinatal death of a preterm baby from a twin pregnancy (Fig. 1). Table III describes the perinatal outcome with regard to the absence or presence of HCV RNA in seminal plasma and to the co-infection by HIV of the male partner. The presence of HCV RNA in seminal plasma was found to influence neither the global rate of pregnancy nor the number of births. Conversely, the rate of gestation, the birth rate and the number of preterm babies were significantly higher (P < 0.05, 0.05 and 0.01, respectively) in the couples where the male partner was co-infected by HCV (Table III). With regard to the ART that led to the occurrence of a gestation event, IUI was used in 6 of the 9 HIV co-infected couples (66.7%) and in only 6 of the 27 HCV infected ones (22.2%) (P < 0.05 by Fisher’s exact test).

The 27 living babies resulting from 24 pregnancies (20 singletons and 7 twins) were tested for HCV antibodies—and for HIV antibodies in case of father co-infection—within the 3 months following birth. All the serological results were negative for these two markers.

### Table II

**Table II Semen characteristics of the male partner and ART results for IVF and ICSI of the female partner, according to the presence or absence of HCV RNA in seminal plasma**

<table>
<thead>
<tr>
<th>HCV RNA in seminal plasma*</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen characteristics in men</td>
<td>n = 64</td>
<td>n = 22</td>
</tr>
<tr>
<td>Mean [sd] ejaculate volume/ml</td>
<td>3.21 [1.54]</td>
<td>3.37 [1.41]</td>
</tr>
<tr>
<td>Mean [sd] spermatozoon number/ml × 10⁶</td>
<td>37.91 [62.16]</td>
<td>24.58 [32.61]</td>
</tr>
<tr>
<td>Mobility rate (%) of spermatozoa [sd]</td>
<td>46 [14.84]</td>
<td>42.79 [11.91]</td>
</tr>
<tr>
<td>Abnormal morphology rate (%) of spermatozoa [sd]</td>
<td>54.78 [19.98]</td>
<td>55.64 [16.86]</td>
</tr>
<tr>
<td>Mean [sd] leucocyte number/ml × 10⁶</td>
<td>0.53 [1.51]</td>
<td>1.38 [3.01]</td>
</tr>
<tr>
<td>ART parameters for IVF and ICSI in women n</td>
<td>n = 30</td>
<td>n = 15</td>
</tr>
<tr>
<td>Number of aspirations</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Mean [sd] oocyte number per aspiration</td>
<td>10.8 [7]</td>
<td>11.7 [7]</td>
</tr>
<tr>
<td>Rate (%) of top-quality embryos</td>
<td>62.4</td>
<td>57.8</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>58.4</td>
<td>63.0</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%) / aspiration</td>
<td>25.0</td>
<td>26.5</td>
</tr>
<tr>
<td>Take home baby rate (%) / aspiration</td>
<td>21.2</td>
<td>20.6</td>
</tr>
</tbody>
</table>

**ART**: assisted reproductive technique; **HCV**: hepatitis C virus; **ICSI**: intracytoplasmic sperm injection; **IVF**, in vitro fertilization.
*No statistically significant difference at the 5% level was observed between the two groups for any of the listed parameters by using tests adapted to small effectives (Student’s t-test for mean comparisons and Fisher’s exact test for qualitative variables).

### Discussion

This prospective study conducted in HCV serodiscordant infertile couples involved in an ART programme was undertaken to extensively explore the impact on ART of the presence of HCV RNA in seminal plasma and the risk of HCV transmission to the newborn.

First, paired-blood and seminal samples from men chronically infected by HCV, including sequential samples in some of them, were systematically tested, leading to a better understanding of the shedding of HCV in the genital compartment of infected men.
A similar prevalence of HCV RNA in seminal plasma was found for HCV and HIV-HCV co-infected men, in agreement with the results of Halfon et al. (2006) but in contradiction with those of other studies that suggested a higher rate of HCV RNA in seminal plasma of co-infected men (Leruez-Ville et al., 2000; Briat et al., 2005). These differences are probably due to the fact that the populations studied did not exhibit the same characteristics in terms of age, mode of contamination, sexual behaviour, clinical stage for HIV or HCV and antiretroviral therapy. In the latter reports, most of the subjects were HIV/HCV-co-infected men having sex with men. As reported previously by our group and others, a positive correlation was observed between the presence of HCV RNA in semen and a high viral load in blood (Bourlet et al., 2002; Pekler et al., 2003; Briat et al., 2005). When sequential samples were available, an intermittent excretion of HCV RNA in semen was observed in around one-half of the subjects (Table I), probably due to the low viral load in semen, close to the threshold of detection of the more sensitive assays, and to the variations of the seminal characteristics from different ejaculates of a same individual. The results depicted in Table I illustrate another important finding: the absence of detection of HCV RNA in a single sample of seminal plasma cannot exclude an intermittent shedding of viral genome in this compartment, so that a negative test on one day does not mean that it will not be positive on another day. In addition, owing to the sensitivity of the assay, a sample can never be categorized as being absolutely negative. Consequently, the recommendation to routinely analyse the seminal

---

Table III  Perinatal outcome and infant characteristics at birth as a function of absence or presence of HCV RNA in the semen and co-infection by HIV of the male partner

<table>
<thead>
<tr>
<th></th>
<th>HCV RNA in seminal plasma</th>
<th>Serological status of the male partner</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 22)</td>
<td>Negative (n = 64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
<td>Newborn</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 (54.5)</td>
<td>8 (36.4)</td>
<td>20 (31.2)</td>
</tr>
<tr>
<td></td>
<td>24 (37.5)</td>
<td>20 (31.2)</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Singleton</td>
<td>4 (18.2)</td>
<td>16 (25.0)</td>
</tr>
<tr>
<td></td>
<td>2 (9.1)</td>
<td>2 (9.1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2 (9.1)</td>
<td>2 (9.1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Twin</td>
<td>2 (9.1)</td>
<td>21* (27.6)</td>
</tr>
<tr>
<td></td>
<td>7 (10.9)</td>
<td>7 (10.9)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Twin</td>
<td>2 (9.1)</td>
<td>21* (27.6)</td>
</tr>
<tr>
<td></td>
<td>7 (10.9)</td>
<td>7 (10.9)</td>
<td>12</td>
</tr>
</tbody>
</table>

Results are expressed as absolute number (percentage). HCV: hepatitis C virus; HIV: human immunodeficiency virus.

*P < 0.01 by Fisher’s exact test between HCV-infected and HIV/HCV-infected male partners.

†P < 0.05 by Fisher’s exact test between HCV-infected and HIV/HCV-infected male partners.

Figure 1  Outcome of ART for the 86 couples enrolled into the study. ART: assisted reproductive technique; IVF: in vitro fertilization (IVF); ICSI: intracytoplasmic sperm injection; FET: frozen embryo transfer; IUI: intrauterine insemination.
plasma from HCV chronically infected men for the presence of HCV RNA before starting an ART programme, loses a great part of its value.

In addition, the key point for a putative transmission of HCV through ART procedures is the presence of HCV RNA in the final prepared fraction of spermatozoa. In contrast to seminal samples (Bourlet et al., 2003), no standardized protocol has been proposed and evaluated prospectively for the detection of HCV RNA in the final spermatozoon fraction. The original technique used in this study, combining the detection of HCV RNA and the amplification of the protamine gene as an external control, allowed us to assess the absence of HCV RNA in 153 final spermatozoon fractions from 86 subjects. To our knowledge, except in the case report from our group (Bourlet et al., 2002), the presence of HCV RNA in such a fraction has never been reported. As suggested previously, this finding emphasizes the efficacy of a semen treatment combining a density-gradient separation and successive washings to eliminate the risk of HCV transmission through ART (Pasquier et al., 2000; Garrido et al., 2004; Garrido et al., 2005; Halfon et al., 2006).

Although the small sample size may impair the power of the comparison, another interesting point documented by this study is that, except for a small non-significant effect on the leucocyte count, the presence of HCV RNA in seminal plasma had no influence on semen parameters (Table II). These results support the findings of Garrido et al. (2005) that HCV plays no role in male infertility. In addition, the presence of HCV RNA in seminal plasma was shown to have no influence on the ART parameters and the pregnancy outcome (Table III). It is worth noting that the pregnancy rate and the number of preterm babies were significantly higher in couples in which the male partner was co-infected by HIV. The first observation is probably related to the fact that ART procedures were proposed to this group of subjects to circumvent the risk linked to HIV and not for infertility purposes, as illustrated by the observation that IUI was the ART that led to pregnancy in a statistically higher number of HIV co-infected couples than of HIV infected ones. The high proportion of premature babies (50%) in the HIV group is more intriguing and its interpretation would need further investigation.

To assess the safety of the procedures evaluated in this study with regard to HCV transmission through ART, an important issue was the systematic evaluation of the HCV status of babies at birth. None of the 27 living newborns were positive for HCV (or HIV in case of father’s co-infection). Interestingly, six of them were conceived with spermatozoon cells purified from semen found positive for HCV RNA. Similarly, no HCV contamination has been reported among the previously published cases (Cassuto et al., 2002; Levy et al., 2002; Sifer et al., 2003; Garrido et al., 2004).

Concerning the protocols used for ART, a large percentage of ICSI was performed (Fig. 1). In most cases, ICSI was chosen because of the necessity to use frozen–thawed semen, since freezing sperm results in decreased mobility, especially in subjects with semen of poor quality (Marcus-Braun et al., 2004). In other cases, ICSI was performed because of the failure of other ART attempts, which could contribute to an explanation for the high rate of miscarriage noted in this study. Also, in some cases, there may have been a delay in the use of ART since, until 2001, it was not possible to propose ART to people infected by HCV in France. The efficiency of ICSI performed in this study was compared with that reported by the French National Register (FIVNAT), which collects the ART data from all French infertility centres (ESHRE, 2006). The pregnancy rate per aspiration was 25.6% in our study and 23.4% as reported by FIVNAT, confirming that a positive HCV serological status did not influence the ART efficiency. Despite its increasing use in ART (Jain and Gupta, 2007), ICSI has been recently associated with a higher risk of major congenital anomalies (Sanchez-Albisua et al., 2007; Sarkar, 2007). Removal of the procedure for the detection of HCV RNA in seminal plasma, as supported by the results of this study, would allow the removal of the sperm-freezing step. Consequently, in couples where the male partner is infected by HCV, the indication for the ART would not be further conditioned by viral considerations.

We acknowledge that only 22 of the study population exhibited positive HCV RNA in seminal plasma and were therefore at higher potential risk of transmitting HCV through ART; however, as the detection of HCV RNA in seminal plasma is intermittent, it could not be excluded that a small risk of transmission remained with sperm samples taken from HCV chronically infected men who tested negative for HCV RNA in seminal plasma at the time of ART. Nevertheless, the present study demonstrates retrospectively the efficiency of sperm washing to eliminate HCV RNA from spermatozoa, the absence of any influence of seminal HCV infection on semen and ART parameters, or on pregnancy outcome, and the absence of HCV infection in the 27 living babies obtained after medically assisted procreation. In conclusion, despite the small size of the study population, these results suggest that there is no need for couples where the male partner is chronically infected by HCV and entering an ART programme to be tested for the presence of HCV RNA in seminal plasma, as presently required by the French legislation. Conversely, the systematic testing of the newborn for HCV antibodies within the first 3 months of life is recommended in order to retrospectively control the safety of ART.

Authors’ contribution

Acknowledgements
We are grateful to Catherine Soler for excellent technical assistance and to Arnauld Garcin from the Direction de la Recherche Clinique of the University Hospital of Saint-Etienne for his help in the collection of clinical data. We are indebted to Philip Lawrence for having improved the quality of the language of the manuscript.

Funding
This work was supported by a grant of the French Ministry of Health (Programme Hospitalier de Recherche Clinique Régional 2001, 0108062).
References


Submitted on October 27, 2007; resubmitted on August 12, 2008; accepted on August 20, 2008.

