



ORIGINAL ARTICLE

# Enhanced Type III Secretion System Expression of Atypical *Shigella flexneri* II:(3)4,7(8)

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**Abstract**

**Objectives:** We aimed at evaluating the virulence of atypical *Shigella flexneri* II:(3)4,7(8) by DNA microarray and invasion assay.

**Methods:** We used a customized *S. flexneri* DNA microarray to analyze an atypical *S. flexneri* II:(3)4,7(8) gene expression profile and compared it with that of the *S. flexneri* 2b strain.

**Results:** Approximately one-quarter of the atypical *S. flexneri* II:(3)4,7(8) strain genes showed significantly altered expression profiles; 344 genes were more than two-fold upregulated, and 442 genes were more than 0.5-fold downregulated. The upregulated genes were divided into the category of 21 clusters of orthologous groups (COGs), and the “not in COGs” category included 170 genes. This category had virulence plasmid genes, including the *ipa-mxi-spa* genes required for invasion of colorectal epithelium (type III secretion system). Quantitative reverse-transcription polymerase chain reaction results also showed the same pattern in two more atypical *S. flexneri* II:(3)4,7(8) strains. Atypical *S. flexneri* II:(3)4,7(8) showed four times increased invasion activity in Caco-2 cells than that of typical strains.

**Conclusion:** Our results provide the intracellularly regulated genes that may be important for adaptation and growth strategies of this atypical *S. flexneri*.

## 1. Introduction

*Shigella* spp. is the causative agent of bacillary dysentery in humans. It is transmitted via the fecal–oral route and causes disease by invading the colonic

epithelium, which results in tissue destruction and massive inflammation [1].

The annual incidence of shigellosis had been estimated to be about 10 cases before 1997, which exploded to about 1000–2500 cases during 1998–2000 in Korea.

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Furthermore, the annual incidence of shigellosis is increasing gradually in Korea [2]. Three types of serotypically atypical *Shigella flexneri* strains were isolated in 2007 in our previous study. Among these, the major atypical strain displayed a plural agglutination pattern by reacting with one typing sera (II) and bound with two grouping sera (3)4 and 7(8) (Supplementary Table 1). This atypical strain, II:(3)4, 7(8), showed higher antibiotic resistance to ampicillin, streptomycin, and trimethoprim–sulfamethoxazole than that of typical *S. flexneri*, and its resistance is increasing [3,4].

All pathogenic *Shigella* strains possess a virulence plasmid that encodes the invasion plasmid antigens IpaACDB and the Mxi-Spa-type III secretion system (TTSS) required for invasion of colorectal epithelium [5,6]. The type III secretion pathway is present in numerous Gram-negative pathogenic bacteria to deliver virulence proteins to the host cell membrane [7,8]. The type III secretion system consists of the following: (1) a secretion apparatus that spans the bacterial envelope; membrane expression of *ipa* (Mxi) MxiG to MxiM; surface presentation of *ipa* (*spa*) Spa13, 24, 29, 32, 33, 40, etc.; (b) translocators and effectors that are inserted into the host cell membrane and act as effectors manipulating host cell processes in favor of the bacteria; invasion plasmid antigens (Ipa) IpaA to IpaD, IpgB1, and IpgD [9,10]; (c) chaperones that associate with the translocators *ipgA*, *ipgC*, *ipgE*, and *spa15*; and (d) transcriptional activators required for the expression of components of the type III secretion apparatus: VirB and MxiE [11–14].

Regulation of type III secretion is tightly controlled by the regulators *virF* and the MxiE regulon, and the pCP301 maintenance system as well as two component systems [15,16]. This type III secretion system is only weakly active during bacterial growth in broth and is activated upon contact of the bacteria with host cells [17].

Atypical *S. flexneri* II:(3)4,7(8) also has the Ipa and Mxi-Spa-type III secretion system, but the expression intensity and regulation are still unclear. Consequently, the major aim of this study was to investigate the gene expression profile of atypical *S. flexneri*.

It would be useful to characterize the isolated atypical *S. flexneri* in relation to infections. Additionally, these strains were isolated from children and adults with severe dysentery, emphasizing the need to study these isolates in detail.

## 2. Materials and Methods

### 2.1. Sample collection

The tested atypical *S. flexneri* II:(3)4,7(8) strain was collected from the Korea National Institute of Health. The *S. flexneri* 2b (ATCC12022) strain was purchased from the American Type Culture Collection (ATCC,

Manassas, VA, USA). Serotyping of the *S. flexneri* strains was confirmed using a commercially available antisera kit (Denka Seiken, Tokyo, Japan) specific for all type- and group-factor antigens. Strains were subcultured on tryptic soy agar (TSA) (Difco, Becton Dickinson and Company, Sparks, MD, USA) plates, and serological reactions were performed after about 18 hours of incubation using the slide agglutination test, according to the manufacturer's instructions.

### 2.2. DNA microarray and bacterial RNA isolation

We used a customized *S. flexneri* DNA microarray from eBiogene (Seoul, Korea). This *S. flexneri* microarray allowed us to study the expression of 4670 *S. flexneri* open reading frames. Bacterial RNA was extracted from atypical *S. flexneri* II:(3)4,7(8), and RNA of the *S. flexneri* 2b strain grown on TSA for 18 hours was extracted using a Qiagen total RNA purification kit (Qiagen, Valencia, CA, USA). Bacterial RNA was further purified by DNase I treatment and phenol–chloroform extraction. The size of the RNA was confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### 2.3. cDNA microarray

The integrity of bacterial total RNA was checked by capillary electrophoresis with an Agilent 2100 bioanalyzer and further purified with an RNeasy mini kit (Qiagen). cDNA probes for the cDNA microarray analysis were prepared by reverse transcription of total RNA (25 µg) in the presence of aminoallyl-dUTP and 6 µg of random primers (Invitrogen, Carlsbad, CA, USA) for 3 hours. The cDNA probes were cleaned up using a Microcon YM-30 column (Millipore, Bedford, MA, USA), followed by coupling of Cy3 dye (for reference) or Cy5 dye (for test sample) (Amersham Pharmacia, Uppsala, Sweden). The Cy3- or Cy5-labeled cDNA probes were purified with a QIAquick polymerase chain reaction (PCR) purification kit (Qiagen). Dried Cy3- or Cy5-labeled cDNA probes were resuspended in a hybridization buffer containing 30% formamide, 5× SSC, 0.1% SDS, and 0.1 mg/mL salmon sperm DNA. The Cy3- or Cy5-labeled cDNA probes were mixed and hybridized to a microarray slide (*S. flexneri* microarray) (MYcroarray, Ann Arbor, MI, USA). The slide was washed after an overnight incubation at 42 °C and then dried by centrifugation at 650 rpm for 5 minutes. The hybridization image on the slide was scanned with an Axon 4000B instrument (Axon Instruments, Union City, CA, USA).

### 2.4. Data acquisition and microarray data analysis

After hybridization, the slide was washed and scanned using a GenePix 4000A scanner (Axon Instruments).

Fluorescent spots and local background intensities were quantified using Genepix Pro 3.0 software (Axon Instruments) to obtain gene expression ratios (reference vs. test sample). Global lowness was used for data analysis with normalization. The benchmarks for up- and down-regulated genes in hybridizations were two- and 0.5-fold, respectively. Gene clustering by gene function was performed at the National Center for Biotechnology Information website by clusters of orthologous groups (COGs) of proteins, <http://www.ncbi.nlm.nih.gov/COG> [18].

### 2.5. Quantitative real-time reverse-transcription polymerase chain reaction

Microarray data were confirmed by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) using a LightCycler (Roche, Laval, QC, Canada) with software version 5.32 and a Light-Cycler FastStart DNA MasterPlus Syber Green I Master Mix kit (Roche). Total RNA (10 ng) was converted to cDNA using a Qiagen cDNA synthesis kit (Qiagen), following the manufacturer's instructions. The cDNA was then used as a template for PCR (*Taq* DNA polymerase, Qiagen). The primers used in the RT-PCR assays are listed in [Supplementary Table 2](#).

Crossing points were estimated using the "Fit Points Method" software option. Estimates of fold induction were obtained from triplicate samples using RelQuant software (Roche).

### 2.6. Bacterial infection of cultured cells

Human HeLa cervical epithelial and human Caco-2 colonic epithelial cell lines were grown in Dulbecco's minimal essential medium. HeLa and Caco-2 cells were seeded at  $4 \times 10^5$  cells in six-well plates containing 10% fetal bovine serum. They were infected with three atypical and three typical 2b strains, including ATCC12022 at a multiplicity of infection of  $\sim 100$  per cell, and the plates were centrifuged at  $600 \times g$  for 10 minutes to synchronize the infection stage. After a 1-hour incubation, gentamicin (50  $\mu\text{g}/\text{mL}$ ) was added to kill extracellular bacteria, and the cells were then incubated for 1 hour. The cells were washed five times with phosphate buffered saline (PBS) to eliminate viable extracellular bacteria, and trypsinized and lysed in a solution of 0.5% sodium deoxycholate in distilled water. Dilutions of this suspension were then plated onto brain heart infusion agar, and colonies were counted after overnight growth. The number of bacteria per infected cell was counted and averaged. Experiments were repeated at least three times for each strain tested.

## 3. Results

### 3.1. Global transcription profiles

Comparison of the expression profiles of atypical *S. flexneri* II:(3)4,7(8) was performed with the typical 2b

transcriptome as a control. In total, 4670 expressed genes were categorized by COG of the proteins. We specifically analyzed more than two-fold changed genes. A total of 344 *S. flexneri* II:(3)4,7(8) genes were more than two-fold upregulated, and 442 genes were 0.5-fold downregulated. These up- and downregulated genes were distributed in 12 COG functional categories ([Figure 1A](#) and [B](#)). A major category of the upregulated genes was "not in COGs", which mainly contained *ipa-mxi-spa* virulence plasmid genes. The second category was "replication and recombination". The remaining 10 categories showed minor fold changes. Of the down-regulated genes, a major category was also "not in COGs", but it contained only a few virulence genes. The remaining 11 COG distribution categories showed no specific differences.

### 3.2. Expression of virulence plasmid coding genes

We focused on the "not in COGs" (including virulence plasmid genes) category of the 344 upregulated atypical *S. flexneri* II:(3)4,7(8) genes, specifically the 41 virulence plasmid coded genes. Most of the genes were invasion-related genes, such as *ipa-mxi-spa*. Therefore, we specifically surveyed the major regulatory factors of the virulence genes, such as *virF*, *virB*, *MxiE*, the two-component signal transduction system, and the global transcription regulators [13] ([Table 1](#)).

### 3.3. Expression of *virF* invasion genes

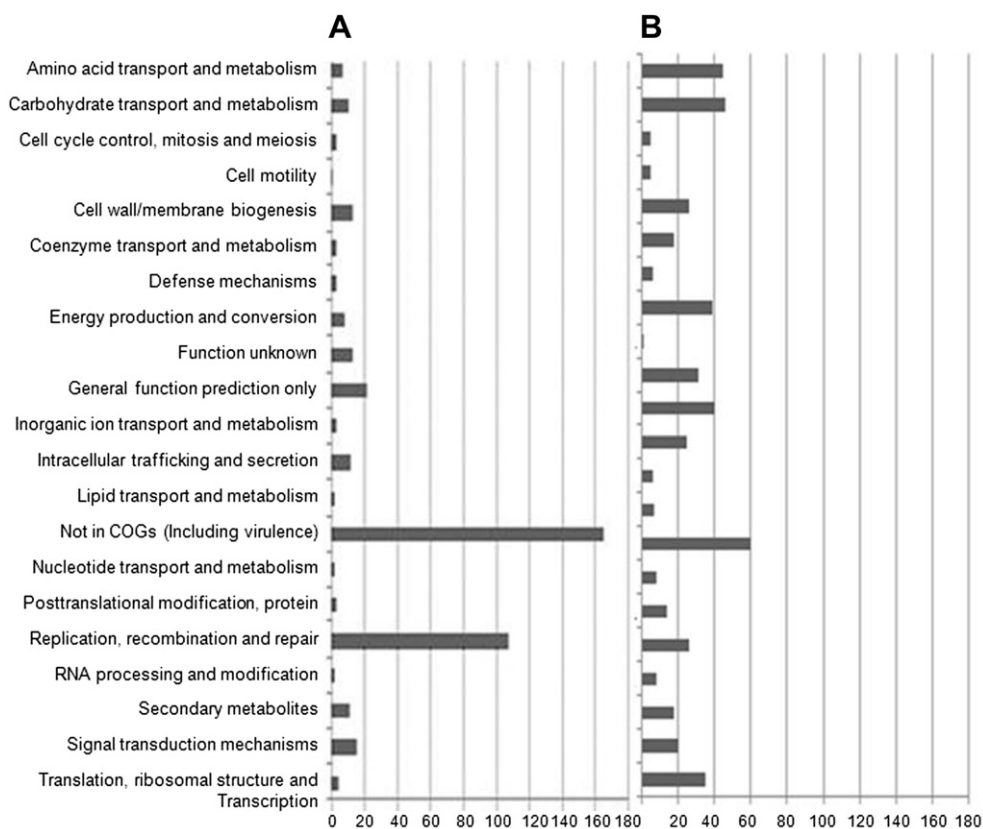
The expression of *ipa-mxi-spa* genes was drastically upregulated in atypical *S. flexneri* II:(3)4,7(8). The *ipa-mxi-spa* expression involves a complex regulatory mechanism [19]. Two plasmid-borne genes, *virF* and *virB*, encode essential regulatory proteins. The VirF protein, an AraC-like transcription factor, activates *virB* and *icsA/virG*, and the VirB protein, in turn, binds to the promoters of entry genes and activates them [20]. *virF* and *virB* expression was observed, and *virF* and *virB* showed 52- and 96-fold increases, respectively, compared to that of the ATCC 2b strain.

### 3.4. Expression of the MxiE regulon

MxiE is a member of the AraC family of regulators that controls a set of late gene products secreted through the Mxi-Spa TTSS [13]. The MxiE regulon is upregulated after entry into epithelial cells and includes *virA*, *ipaH<sub>7,8</sub>* (involved in the escape of *Shigella* into the host cytosol), and *ipaH<sub>9,8</sub>* (the product of which is targeted to the host nucleus) [21]. MxiE showed the highest expression (140-fold increase), and *virA* expression was 14.1-fold upregulated.

### 3.5. Maintenance of virulence plasmid pCP301

The R100 plasmid is the primary replication system of the virulence plasmid, which belongs to the RepFIIA family of replicons [16]. There are also multiple loci on



**Figure 1.** Global transcription profiles of an atypical *Shigella flexneri* strain. (A) More than two-fold upregulated genes were distributed as COGs. (B) More than two-fold downregulated genes were distributed as COGs. X axis indicates the number of genes. COGs = clusters of orthologous groups.

pCP301 that are homologous to sequences known to be involved in plasmid segregation and stable maintenance such as *stbA* and *stbB* of plasmid R100 and *mvpA* and *mvpT*. Among these, *mvpA* and *mvpT* form an effective

system to maintain stability *in vitro* [15]. The expression of *mvpA* was not significantly altered (1.2-fold change), whereas *mvpT* increased 26-fold compared to that in the control strain.

**Table 1.** Gene products influencing expression of *Shigella flexneri* plasmid-linked virulence genes

Gene	Description	Array	RT-PCR
<i>virF</i>	AraC-like transcription regulator; activates transcription of <i>virB</i> and <i>icsA</i> ( <i>virG</i> )	52	54
<i>virB</i>	VirF-dependent vassal regulator; activates main virulence structural gene operons	96	90
<i>mxiE</i>	AraC-like protein	140.5	108
<i>virA</i>	Secreted by the Mxi-Spa secretion	14.1	17
<i>stbA/B</i>	Concerned plasmid segregation and stable maintenance	2/56	1.5/58
<i>mvpA/T</i>	Concerned plasmid segregation and stable maintenance	1.2/26	1.1/30
<i>cpxA/cpxR</i>	Sensor (CpxA)-response regulator (CpxR) two-component system	1.4/0.5	1.2/0.6
<i>phoP/phoQ</i>	transcriptional regulatory protein/sensor protein PhoQ	0.9/0.6	0.1/0.5
<i>envZ/ompR</i>	Sensor (EnvZ)-response regulator (OmpR) two-component system	1.3/0.2	1.2/0.3
<i>rpoS</i>	Master regulator of general stress	0.3	0.4
<i>hns</i>	Nucleoid-associated protein; repressor of <i>virB</i> transcription	1.1	1.5
<i>ispA</i>	Hydrophobic protein; possible role in cell division	0.7	0.5
<i>mia</i>	tRNA <i>N</i> <sup>6</sup> -isopentyladenosine(i <sup>6</sup> A37) synthetase	0.7	0.5
<i>stpA</i>	Analog if H-NS; can repress virulence gene expression	1.9	1.5
<i>topA</i>	DNA topoisomerase type I; relaxes negatively supercoiled DNA	0.5	0.5
<i>parC/parE</i>	DNA topoisomerase type II; decatenase, relaxes negatively supercoiled DNA	0.9/1.2	0.8/1.1

RT-PCR = reverse-transcription polymerase chain reaction.

### 3.6. Two-component signal transduction systems, sigma factors, and stress response factors

Two-component signal transduction systems play an important role in regulating virulence in *S. flexneri*. CpxR binds to the *virF* promoter region when it is phosphorylated by CpxA [22]. Expressions of *cpxA* (1.4-fold increase) and *cpxB* (0.5-fold decrease) were not significantly changed.

The well-characterized PhoP–PhoQ system is crucial for persistent infection and resistance to killing by cationic polypeptides derived from polymorphonuclear leukocytes and other sources [23]. We observed the transcription of these and other two-component systems; PhoP changed 0.8-fold and PhoQ 0.6-fold. Changes were observed for the *ompR*–*envZ* osmosensor system, as *ompR* changed 0.2-fold and *envZ* 1.3-fold.

Expressions of the *rpoS* gene, the master regulator of general stress, and sigma factor were not altered significantly.

H-NS, global regulatory factor *hns* represses the *virB* gene antagonistically. A footprint extending from position –20 to position +20 has been detected *in vitro* at the *virB* promoter, suggesting that H-NS may exclude RNA polymerase from binding there [24,25]. The *hns* changed 1.16-fold and its analog, *stpA*, changed 1.89-fold.

### 3.7. Other regulators

It appeared that the expression of the virulence genes may be coupled to the bacterial cell cycle, as *ispA*, a cell division gene, changed 0.68-fold [26].

The *mia* gene, which codes for the tRNA<sup>N<sup>6</sup></sup>-isopentyladenosine (i<sup>6</sup>A<sup>37</sup>) synthetase, is required for production of the modified nucleoside 2-methyl-N<sup>6</sup>-isopentyladenosine necessary for expressing the virulence regulon [27]. This gene changed 0.74-fold.

It seemed that the alteration in DNA topology facilitated a productive interaction between the DNA-bound VirF protein and RNA polymerase due to changes in pH, temperature, and osmolarity. Thus, the change in DNA supercoiling may promote VirF oligomerization on the DNA, and this may represent an activation step analogous to arabinose binding to the AraC protein. Thus, *topA* (0.56-fold change) [28], the gene encoding DNA topoisomerase I, and *parC* (0.95) and *ParE* (1.16), the genes encoding DNA topoisomerase IV, facilitate virulence gene expression [29]. All these regulators changed <2-fold.

### 3.8. Validation of the microarray results by qRT-PCR

We confirmed the array experiment by qRT-PCR. Regulatory genes of type III secretion systems were selected and compared. RNA samples were isolated

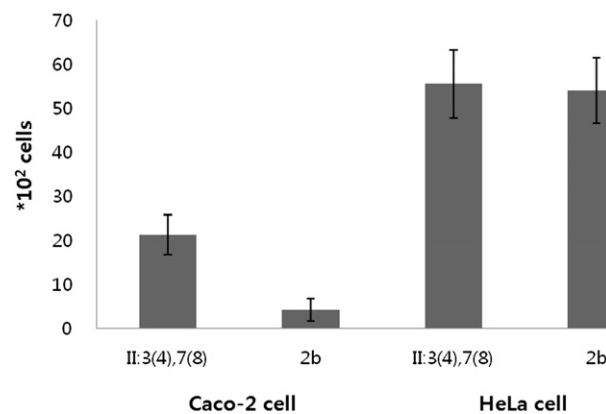
under the same conditions as the microarray experiment, and the qRT-PCR results were compared with the microarray data (Table 1). The qRT-PCR results showed a similar pattern as that of the array experiment. We also tested two more atypical strains to confirm high expression of the type III secretion system. The results showed the same pattern. The primers used are listed in Supplementary Table 2.

### 3.9. Bacterial infection of cultured cells

We questioned the invasion ability of these atypical strains based on the increased expression of the type III secretion system. Invasion of three atypical *S. flexneri* II:(3)4,7(8) and three typical 2b (including ATCC 12022) were estimated in colon carcinoma Caco-2 cells and epithelial HeLa cells. The atypical *S. flexneri* II:(3)4,7(8) strains demonstrated more than four-fold increased ability of invasion compared to that of typical 2b strains, specifically in Caco-2 cells but not in HeLa cells (Figure 2).

## 4. Discussion

In this study, we analyzed the gene expression profile of atypical *S. flexneri* II:(3)4,7(8) and compared it to that of ATCC 2b as a control strain. We analyzed more than two-fold changed genes. Of the two-fold upregulated genes, the major category of 344 genes was “not in COGs”. The “not in COGs” (including virulence plasmid genes) category contained 41 invasion-related genes of the *ipa-mxi-spat* type III secretion system. Among the regulatory genes for the type III secretion system, *virF* and the MxiE regulon were specifically upregulated in atypical *S. flexneri* II:(3)4,7(8). We also confirmed expression of these regulator genes by qRT-PCR in two more atypical *S. flexneri* II:(3)4,7(8) and two more 2b serotype strains. Additionally, atypical



**Figure 2.** Invasion assay for the atypical and typical strains. The Y axis indicates ×10<sup>2</sup> bacteria.

*S. flexneri* II:(3)4,7(8) showed a four-fold increased invasion activity in Caco-2 cells compared to that of typical strains but not in HeLa cells. In the human colonic epithelial cell line Caco-2, which differentiates into a polarized epithelium expressing a well-established brush border, the invasion process occurs through basolateral surfaces and has been used in an invasion study [30,31].

The type III secretion system is regulated at several levels. First, transcription of secretion genes is regulated by global regulators, such as H-NS, IspA, Mia, ParC, and ParE, which respond to changes in temperature, pH, or osmolarity [32]. These global regulators did not show specific changes in the atypical *S. flexneri* II:(3)4,7(8) strains. Thus, increased expression of the type III secretion system in atypical strains was low in relation to global regulation. Next, activity of the type III secretion system apparatus is regulated in response to specific stimuli, including contact with host cells, exposure to artificial compounds such as Congo red, and alterations in the growth environment. These stimuli were not altered in atypical *S. flexneri* II:(3)4,7(8) strains or in the control strain. Thus, various stimuli did not increase expression of the type III secretion system in the atypical strains. Finally, transcriptional activators such as VirB and MxiE were required for proper expression of the type III secretion apparatus. The atypical *S. flexneri* II:(3)4,7(8) strains showed more than 100-fold increases in MxiE and a 96-fold increase in VirB expression compared to those of the control strain; thus, increased type III secretion system expression of the atypical strain originated from increased MxiE and VirB transcription. Most *Shigella* spp. show low transcription levels of these regulators and increase only when they contact host epithelial cells or are exposed to artificial compounds such as Congo red. Other reports have shown that increased secretion occurs when some genes such as *ipaB* and *ipaD* are inactivated, resulting in deregulated secretion [17,33]. Inducing or deregulating secretion activity also results in increased transcription of some genes encoding secreted proteins, such as the *virA* and *ipaH* genes in *Shigella* spp. [13]. In atypical *S. flexneri* II:(3)4,7(8), the type III secretion system was induced by an increased expression of the MxiE regulon, but the exact reason for the increased expression of the virulence-related genes is still uncertain in atypical *S. flexneri* II:(3)4,7(8). Increased type III secretion system expression and increased invasion ability of human epithelial cells of these atypical *S. flexneri* II:(3)4,7(8) strains will be studied specifically in the near future, because this atypical *S. flexneri* II:(3)4,7(8) strain is increasing in prevalence in Korea and shows increased antibiotic resistance.

These results of this study will facilitate functional studies of intracellularly regulated genes that may be important for adaptation and growth strategies of this atypical *S. flexneri* during infection.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in online version at <http://dx.doi.org/10.1016/j.phrp.2012.10.002>.

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