

RAPD and ERIC-PCR coupled with HRM for species identification of non-dysenteriae *Shigella* species isolated from clinical specimens; As a potential of alternative method

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Abstract

Background: Species identification of *Shigella* isolates are so prominent for epidemiological studies and implementing infection prevention strategies. We developed and evaluated RAPD and ERIC-PCR assays coupled with HRM for differentiation of non-dysenteriae *Shigella* species as potential alternative methods. Isolation of eighteen *Shigella* strains from faecal specimens collected from children under 2 years old with diarrhea symptom (n=143), species of the isolates were identified by slide agglutination assay as the golden standard method. Species of isolates were identified using developed RAPD-PCR-HRM and ERIC-PCR-HRM assays and differentiation of data sets obtained from difference plots of HRM was implemented by PCA as a dimension reduction method then sensitivity and specificity of the methods were evaluated using ROC curve.

Results: Consequently, we found that RAPD-PCR-HRM with sensitivity and specificity of 100 and 85% respectively is significantly more suitable for identification of non-dysenteriae *Shigella* species. However, sensitivity and specificity of ERIC-PCR-HRM were evaluated 33 and 46% respectively.

Conclusion: Regardless of inherent poor reproducibility of DNA fingerprinting based methods, RAPD-PCR-HRM assay can be considered as a potential of alternative method for species identification. Also, HRM technique used in this study is more rapid, inexpensive, and sensitive than gel electrophoresis for characterization of PCR amplicons.

Background

Shigella is a facultative anaerobe, gram-negative bacilli belonging to the *Enterobacteriaceae* family with intestinal and extraintestinal pathogenicity in human (Shigellosis). Four species (with different serotypes) have been characterized for this genus including: *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae*. *Shigella* species cause mild to severe diarrhea in adults and children (up to 28% fatality rate in children with severe symptoms) as intestinal pathogens; however, *S. dysenteriae* type 1 lead to hemorrhagic uremic syndrome (HUS) because of releasing Shiga-toxin; it is regarded as the only extraintestinal disease caused by this genus [1]. There are significant differences between the severity and type of the gastrointestinal disorders triggered by *Shigella* species. Also, the geographical distributions of prevalence rate of *Shigella* species are considerably varied among developing and industrial countries [2]. Epidemiological studies revealed that identification of *Shigella* species plays a crucial role for providing acceptable clinical investigation and tracing of the outbreaks generated by this pathogen [3]. Therefore; to support the epidemiological investigation of shigellosis outbreaks especially among high-risk population as well as infant cases, different serological, molecular and immunological assays have been developed for identification of *Shigella* species. The most prominent challenge of *Shigella* species identification is differentiating all species from each other [4]. However; differentiation of *Sh. dysenteriae* is more practicable because of harboring *stx* gene as the specific marker [5]. Some of these identification methods consist of DNA hybridization, multiplex PCR, whole genome sequencing, immunocapture PCR, MALDI-TOF mass spectrometry, rRNA gene restriction pattern, and serological methods as the gold

standard. However, many of these methods, despite the optimization, are too expensive and complicated to be employed in clinical labs. Thus, new methods as well as more affordable and user-friendly assays with acceptable specificity and sensitivity are still needed to be developed and optimized [6].

DNA fingerprinting techniques are carried out for characterization and typing of microbial genomes. These methods are divided into two main categories: sequencing-based assays such as multi-locus sequence typing (MLST), repetitive element sequence-based (Rep-PCR), microarray, comparative hybridization, and whole genome sequencing; and non-sequencing-based techniques e.g. random amplification polymorphism DNA (RAPD-PCR), enterobacterial repetitive intergenic consensus (ERIC-PCR), pulsed field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) assays [7]. Obviously, sequence-based typing techniques are more expensive, time consuming, and complicated than other group of assays [8]. DNA PCR fingerprinting methods e.g. RAPD and ERIC have been widely used as genotyping assay for enterobacterial foodborne pathogens. However, these techniques have been developed for species identification of some bacterial and fungal strains regardless of species-specific sequences [9]. Because of the complicated PCR products of RAPD and ERIC-PCR, statistical and mathematical analysis of the results are suggested to be carried out for further genomic characterization and species identification. Also; requiring characterization of the PCR products of RAPD and ERIC-PCR, usually implemented by electrophoresis on agarose gel, is considered as another challenge of these methods for species identification [10].

High resolution melting curve analysis (HRMA) provides characterization of PCR products as a rapid alternative assay of gel electrophoresis with higher precision and differentiation potential of PCR products with the same size [11]. This property of HRMA makes it considerable to be employed for analysis of the complicated PCR products as well as in genotyping and DNA fingerprinting protocols. PCR molecular typing methods such as RAPD coupled with HRMA has been developed successfully to characterize microbial strains in intra-serovar levels and for species identification [12]. However, use of PCR based DNA fingerprinting methods coupled with HRMA despite many advantages for serotype and species identification of microbial strains is limited to a few researches [13]. In the present study, we developed RAPD and ERIC-PCR fingerprinting assay coupled with HRMA following analysis by principal component analysis (PCA) for identification of *Sh. sonnei*, *Sh. flexneri* and *Sh. boydii* strains after isolation from clinical specimens.

Results

So far, the most reliable method for identification of *Shigella* species is the gold standard assay by slide agglutination method using species-specific polyvalent antisera [14]. Despite higher sensitivity and specificity, this method is very expensive so many researchers have made efforts to develop new alternatives. Because of high-cost, complicated, and sensitive production process of monoclonal antibodies in serology-based methods, molecular assays as well as DNA based techniques are preferred to be developed for species identification of bacterial pathogens [15]. However, identification of *S.*

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js e as an individual marker of this pathogen is not

regarded as a real challenge in comparison with identification of other species [16]. At the present study, we isolated and identified *Shigella* species in eighteen specimens (totally from 143 children under 2 years old with gastrointestinal disorder; 12.58%) consisting of 11 *S. sonnei*, 3 *S. boydii*, and 4 *S. flexneri* isolates by golden standard method using culture-based technique for *Shigella* isolation and serologic assay for species identification (Table 1). After isolation by culture-based methods, we evaluated and developed RAPD and ERIC-PCR coupled with HRM methods as sensitive and specific alternative assays for *Shigella* species identification. Normalized and difference melting curves of isolates by RAPD-PCR-HRM are shown in Figs. 1 and 2, respectively. Because of extremely complicated PCR products amplified in RAPD, very complex melting curves were obtained after HRM step which made it impossible to categorize melting profiles by the instrument software algorithm [17]. Therefore; at the present study, we have employed a dimension reduction method, PCA as recommended by many researchers, to simplify analysis of this complex and huge data. PCA assay was performed on the data obtained from the output of HRM difference plot. PC 3D loading plot for difference melting curve data set of RAPD-PCR-HRM in species identification of *Shigella* isolates has been illustrated in Fig. 3. PCA plot showed that three distinctive groups as we considered three different species for *Shigella* isolates. Except one isolate of *S. boydii*, all species of isolated *Shigella* strains were identified correctly corresponded with the golden standard method for species identification by developed RAPD-PCR-HRM assay.

Table 1.

Shigella isolates identified in this study by golden standard methods

Species	Isolate name/ATCC number
<i>S. sonnei</i> (SS1)	SSQI-2020-221
<i>S. sonnei</i> (SS2)	SSQI-2020-222
<i>S. sonnei</i> (SS3)	SSQI-2020-223
<i>S. sonnei</i> (SS4)	SSQI-2020-224
<i>S. sonnei</i> (SS5)	SSQI-2020-225
<i>S. sonnei</i> (SS6)	SSQI-2020-226
<i>S. sonnei</i> (SS7)	SSQI-2020-227
<i>S. sonnei</i> (SS8)	SSQI-2020-228
<i>S. sonnei</i> (SS9)	SSQI-2020-229
<i>S. sonnei</i> (SS10)	SSQI-2020-230
<i>S. sonnei</i> (SS11)	SSQI-2020-231
<i>S. sonnei</i> (Reference-SSR)	ATCC 25931
<i>S. boydii</i> (SB1)	SBQI-2020-443
<i>S. boydii</i> (SB2)	SBQI-2020-444
<i>S. boydii</i> (SB3)	SBQI-2020-445
<i>S. boydii</i> (Reference-SBR)	ATCC 12030
<i>S. flexneri</i> (SF1)	SFQI-2020-328
<i>S. flexneri</i> (SF2)	SFQI-2020-329
<i>S. flexneri</i> (SF3)	SFQI-2020-330
<i>S. flexneri</i> (SF4)	SFQI-2020-331
<i>S. flexneri</i> (Reference-SFR)	ATCC 12022

ERIC-PCR-HRM was evaluated as another assay carried out in this study for species identification of the same *Shigella* strains. Figures 4 and 5 showed normalized and difference plots of ERIC-PCR-HRM assay respectively for *Shigella* isolates. As the same as the fluorescence plots obtained from the RAPD-PCR-HRM; because of extremely varied amplicon size and sequence, high complexity of melting profiles also does not allow normal analysis to be implemented for HRM data set in this procedure [17]. Difference plot output data of ERIC-PCR-HRM was analyzed for principal components determination with PCA. PC 3D loading plot of ERIC-PCR-HRM data set has been shown in Fig. 6. Seven species among eighteen isolates were recognized incorrectly showed that RAPD assay is more sensitive and specific than ERIC-PCR to identify *Shigella* species while they are coupled with HRM and analyzed by PCA. Sensitivity and specificity of an assay as the accuracy characteristics of a screening or identification test relative to a golden standard method are commonly evaluated by ROC curve [18]. ROC curves of the assays developed in this study for *Shigella* species identification including RAPD and ERIC-PCR-HRM assays are illustrated in Figs. 7 and 8, respectively. Sensitivity and specificity of the RAPD-PCR-HRM assay were observed 100 and 85%, respectively. However, sensitivity and specificity of the ERIC-PCR-HRM technique were calculated 33 and 46% respectively regarding significant differences in comparison with the other assay. Nevertheless, these remarkable differences in methodological characteristics were predictable considering the results of PCA for each individual assay.

Discussion

DNA fingerprinting methods recently have been introduced as the assay for the purpose of microbial species identification. For the first time, Tulsiani et al. (2010) used high resolution melting curve analysis of RAPD for characterization of *Leptospira* strains to serovar level. They were successful to develop RAPD-HRM assay as an inexpensive and rapid method to identify different serotypes of *Leptospira*. However, melting profile analysis by Rotor-Gene 6000 series software package was used for clustering of the isolates and differentiating between the serotypes [19]. Miller et al. (2015) developed HRM-based assay for identification of *Pasteurellaceae* species by amplification of varied regions in 16 s rRNA sequence and they found this method sensitive and rapid for species identification [20]. Chen et al. (2019) also employed RAPD-HRM assay for differentiation of pathogenic and non-pathogenic *Escherichia coli* isolates from each other and they found it a sensitive, specific, and practical assay. There are few studies about species identification of bacterial strains using DNA fingerprinting methods coupled with HRM; however, many researchers investigated these methods for differentiating non-microbial species such as raw meat, animals, and plant species especially by RAPD-PCR-HRM assay. Also, RAPD-HRM assay has been used for genotyping of bacterial pathogens previously [17].

High resolution melting curve analysis of ERIC-PCR has not been developed and employed for genotyping and other molecular characterization of microbial genomes yet and the present study is the first one evaluated this method coupled with HRM as a molecular technique for diagnostic purpose [21]. During HRM procedure, saturating dyes bound to DNA then emit fluorescence because of dissociation of double-strand DNA while the temperature is rising gradually then the emission is measured by a highly sensitive molecular assay, it can be found that any

difference as well as a single nucleotide polymorphism in amplicon sequences significantly changes the melting profile [22]. In DNA fingerprinting methods such as RAPD and ERIC-PCR assays, broad range of amplicons are produced throughout the PCR step. These complex PCR products are individually considered as a DNA marker for each organism and can be characterized by different methods [23]. Gel electrophoresis is commonly carried out for characterization of PCR products of DNA fingerprinting assay. HRM as a more rapid, inexpensive, sensitive and user-friendly method is used and more preferred for characterization of PCR products [24]. By HRM, RAPD and ERIC-PCR products are barcoded for better characterization and clustering of isolates in serotype and species identification. Because of the complex properties of these methods in PCR amplicon production, it is unclear that why one method is more practical, sensitive, and specific than the other one in species identification in comparison with the golden standard method [25]. At the present study, we found that RAPD-PCR-HRM assay is more appropriate and acceptable than ERIC-PCR-HRM method for *Shigella* species identification. However, this result may not be obtained for species or serotype identification of other microorganisms in the future studies. ERIC are repetitive sequences located on some specific genomic regions of Enterobacteriaceae family bacteria which are amplified during the PCR reaction [26]. In RAPD-PCR, randomly amplification is performed throughout the microbial genome on a wide range of different DNA regions which can be located on any probable position. Although both methods are based on random amplification, higher variation of amplified genomic regions in RAPD-PCR method may result in more efficient differentiation capability [27]. Limited genomic regions for DNA fingerprinting and barcoding classification makes ERIC-PCR inefficient to discriminate genomic differences among microbial strains and species. As previously investigated by many researchers, discriminatory index of RAPD was significantly higher than that of ERIC-PCR for genotyping of bacterial isolates. It is worthy to note that the most prominent drawback of DNA barcoding and fingerprinting methods based on random primer DNA typing such as RAPD and ERIC-PCR to identify microbial serotype and species is inherent poor reproducibility [28]. This main inherent problem has not been solved yet; also, we have not investigated on this challenge at the present study. However, it is more appreciated and suggested to improve and develop DNA fingerprinting methods with higher reproducibility in future studies. Nevertheless, we introduced RAPD-PCR-HRM assay as a potential alternative method for non-dysenteriae *Shigella* species identification; however, future studies with more sample size, and wide range of different molecular techniques are suggested to be implemented.

Conclusion

DNA fingerprinting methods usually are used for genotyping of microbial pathogens. These methods can be considered as the potential of alternative methods for differentiation of microbial serotypes and species. In this study, we developed and evaluated RAPD and ERIC-PCR methods coupled with HRM assay for identification of non-dysenteriae *Shigella* species isolated from clinical specimens. The authors attempted to analyze the data set obtained from the difference plots of HRM by PCA as a dimension reduction method to simplify the classification of melting profiles. We isolated eighteen *Shigella* strains in faecal specimens collected from children (n = 143) with gastrointestinal disorders and diarrhea symptom then species of the strains were identified by slide agglutination assay as the golden standard

method. Also, species of the isolates were identified by developed RAPD-PCR-HRM and ERIC-PCR-HRM methods analyzed with PCA. We found RAPD-PCR-HRM assay more appropriate than ERIC-PCR-HRM as the potential of alternative method for non-dysenteriae *Shigella* species identification with sensitivity and specificity of 100 and 85%, respectively. Inherent poor reproducibility is the only disadvantage of all DNA fingerprinting based methods such as RAPD for genotyping or species and serotype identification which is suggested to be improved for the future studies.

Methods

Fecal samples collection and reference bacterial strains

Children under 2 years old with diarrhea symptom suspected to gastroenteritis (n = 163) in Qazvin, Iran, during December 2019 to February 2020 were referred to the central laboratory of Qods Children Hospital of Qazvin for microbiological investigation. As previously described by WHO Laboratory investigations manual of Enteric infections for faecal sample preparation, all suspected specimens were collected in disposable and clean containers then examined macroscopically and microscopically for color, mucus, consistency, and red blood cells [14]. All specimens were kept at 4 °C prior to bacterial isolation procedure. We also used *S. sonnei* ATCC 25931; *S. flexneri* ATCC 12022; and *S. boydii* ATCC 12030, purchased from Pasteur institute of Iran (Pasteur institute, Tehran, Iran), as *Shigella* reference strains in this study. All reference strains were activated by inoculation in Bovine Heart Infusion (BHI) broth and incubation at 37 °C overnight.

Shigella isolation and species identification

All specimens were initially cultured on MacConkey agar and Salmonella-Shigella agar (SSA) media (Promedia, Spain) incubated aerobically for 24 h at 37 °C. Non-lactose-fermenting and H₂S-negative colonies on MacConkey agar and SSA were isolated for biochemically identification. Biochemical tests consisted of Urease, Triple Sugar Iron (TSI), Motility, Siminuous Citrate, and Indole tests. Biochemically confirmed colonies were further serologically analyzed for *Shigella* species identification. Serological identification was carried out using slide-agglutination test with DIFCO *Shigella* species specific antisera (Difco Lab, Michigan, USA) as the gold standard assay [15].

Genomic DNA extraction

Bacterial isolates and the reference strains were inoculated into LB broth then incubated at 37 °C overnight. After centrifugation of the incubated LB broth tubes at 6000 RPM for 10 min, the cell palette in each centrifuge tube were subjected to genomic DNA extraction employing Sinaclon gram-negative DNA extraction Kit (Sinaclon, Iran) according to the instructions described by the manufacturer. The quantity and quality of the extracted genome were evaluated using NanoDrop Spectrophotometer (Thermo Scientific, USA). Before PCR-HRM, concentration of all DNA templates was adjusted to 50 ng. µL⁻¹.

RAPD and ERIC-PCR coupled with HRM assay

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In this study, we used the single random oligonucleotide primer 5- *CGCGTGCAG* - 3 for RAPD-PCR and species identification of isolated *Shigella* strains [16]. Each reaction tube contained 20 µL of final volume, including 10 µL of 2X HotStart EvaGreen PCR-HRM master mix (Solis BioDyne Co, Estonia), 0.5 µL of RAPD primer (20 pM), 1 µL of DNA template (50 ng), and distilled deionized water to the final reaction volume. RAPD-PCR coupled with HRM assay was performed using Rotor-Gene 6000 real-time PCR instrument (Corbett, Australia) as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 36 °C for 1 min, and 4 min at 72 °C. Then HRM was performed between 70 and 95 °C with continuous ramping 0.1 °C s⁻¹. Fluorescence of melting profile was normalized between 80 and 95 °C by Rotor-Gene 6000 series software version 1.7 (Corbett, Australia) then normalized and difference melting curves were obtained for each isolate.

For ERIC-PCR amplification two primers have been used including ERIC1R 5- *ATGTGCTCTGGGGATTCAC* - 3 and ERIC2 5- *GTGACTGGGGTGAGCG* - 3 [16]. Both RAPD and ERIC primers used in this study previously were employed by researchers for DNA fingerprinting of enterobacterial pathogens. ERIC-PCR was performed in 20 µL final reaction volume containing 10 µL of 2X HotStart EvaGreen PCR-HRM master mix (Solis BioDyne Co, Estonia), 1 µL of each primer (10 pM), 1 µL of DNA template, and distilled deionized water to 20 µL. Rotor-Gene 6000 real-time PCR instrument (Corbett, Australia) also was employed for ERIC-PCR-HRM procedure. Thermal cycling program was initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min. Afterwards, HRM was carried out from 70 to 95 °C with 0.1 °C s⁻¹ ramping. Also, normalized and difference melting curves finally were achieved from the Rotor-Gene 6000 series software by fluorescence normalization of melting profile between 80 and 95 °C.

Data analysis

All difference curves obtained from Rotor-Gene 6000 series software were exported to Excel (Microsoft Office Excel software, Microsoft Corp., Redmond, USA) files then they were used as the input data of analysis. Principal components analysis (PCA) was employed for analysis and data categorization as a dimension reduction method by SPSS software version 23.0 (SPSS, Inc., Chicago, USA). Also, sensitivity and specificity of the methods have been evaluated using receiver operating characteristic (ROC) curve analysis by SPSS. All measurements and analysis were carried out in triplicates.

List Of Abbreviations

HUS: Hemorrhagic Uremic Syndrome; DNA: DeoxyriboNucleic Acid; rRNA: Ribosomal RiboNucleic Acid; PCR: Polymerase Chain Reaction; MALDI-TOF: Matrix Assisted Laser Desorption/Ionization – Time Of Flight; RAPD: Random Amplification of Polymorphic DNA; ERIC: Enterobacterial Repetitive Intergenic Consensus; AFLP: Amplified Fragment Length Polymorphism; PFGE: Pulsed-Field Gel Electrophoresis; HRMA: High Resolution Melting Analysis; PCA: Principal Component Analysis; 3D: Three Dimensional; ROC: Receiver Operating Characteristic

Declarations

Ethics approval and consent to participate

The study protocol for clinical samples was evaluated, reviewed, and approved by the ethics committee of National Institute for Medical Research Development (IR-NIMAD-REC.1397.204). There is not any medical care or treatment for consideration of informed consent.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interest

The authors declare that they have no competing interest.

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Authors' contributions

BP, AA and AK designed the research project. BP, AKh and LA performed all experiments and analysis. BP wrote the manuscript. AA and AK finalized and reviewed the manuscript. All authors investigated the final draft and approved the manuscript.

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Figures

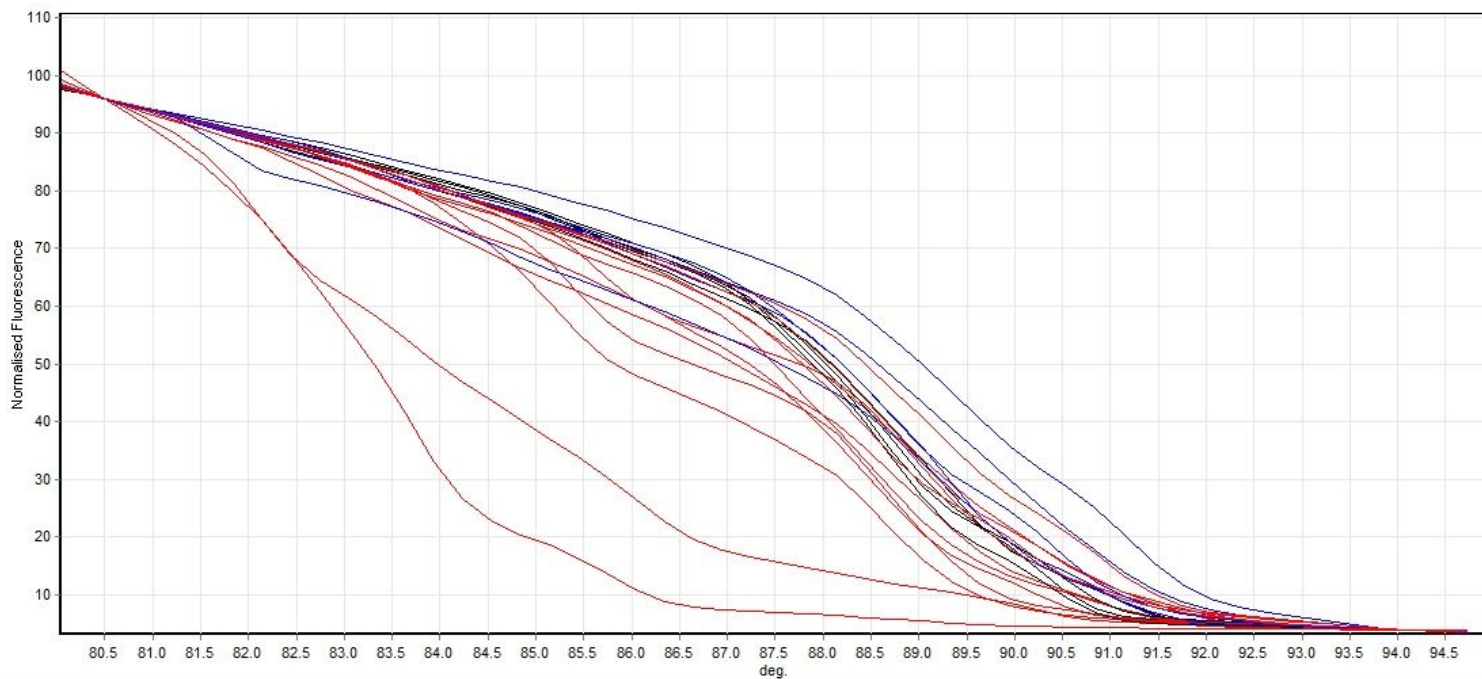


Figure 1

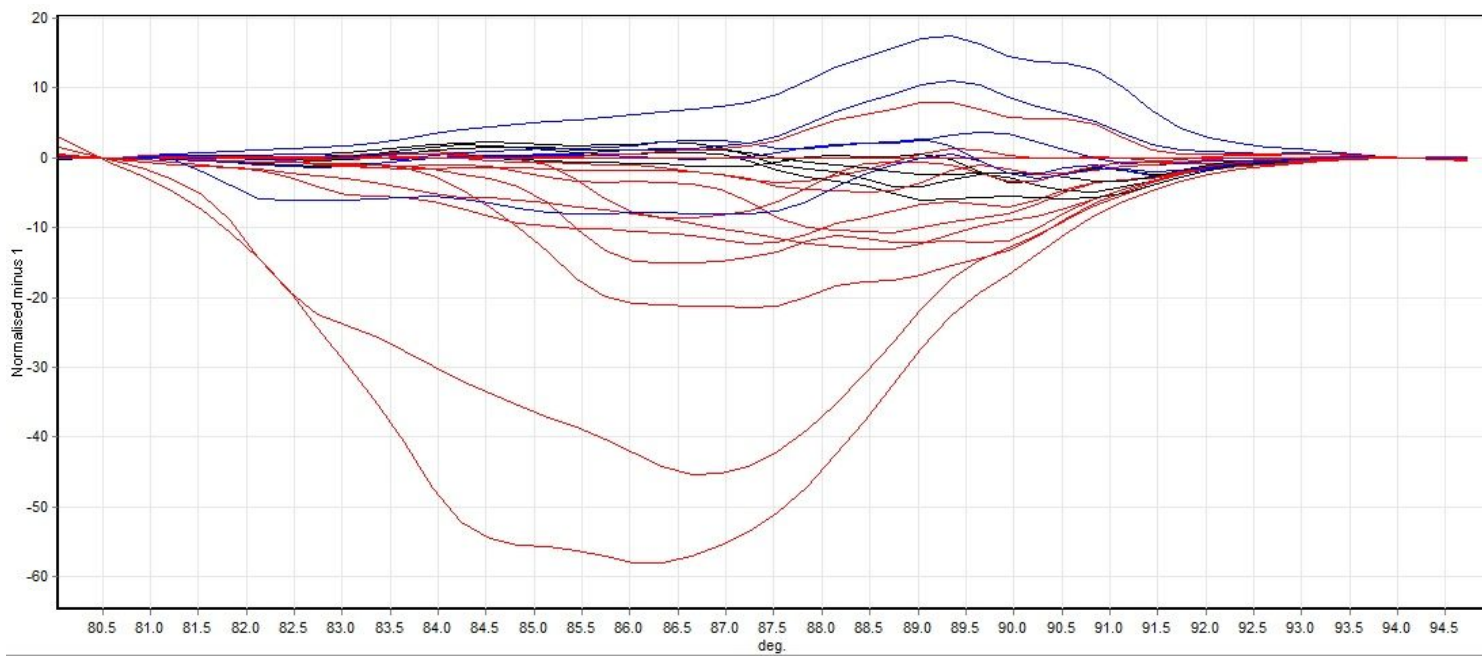


Figure 2

Component Plot

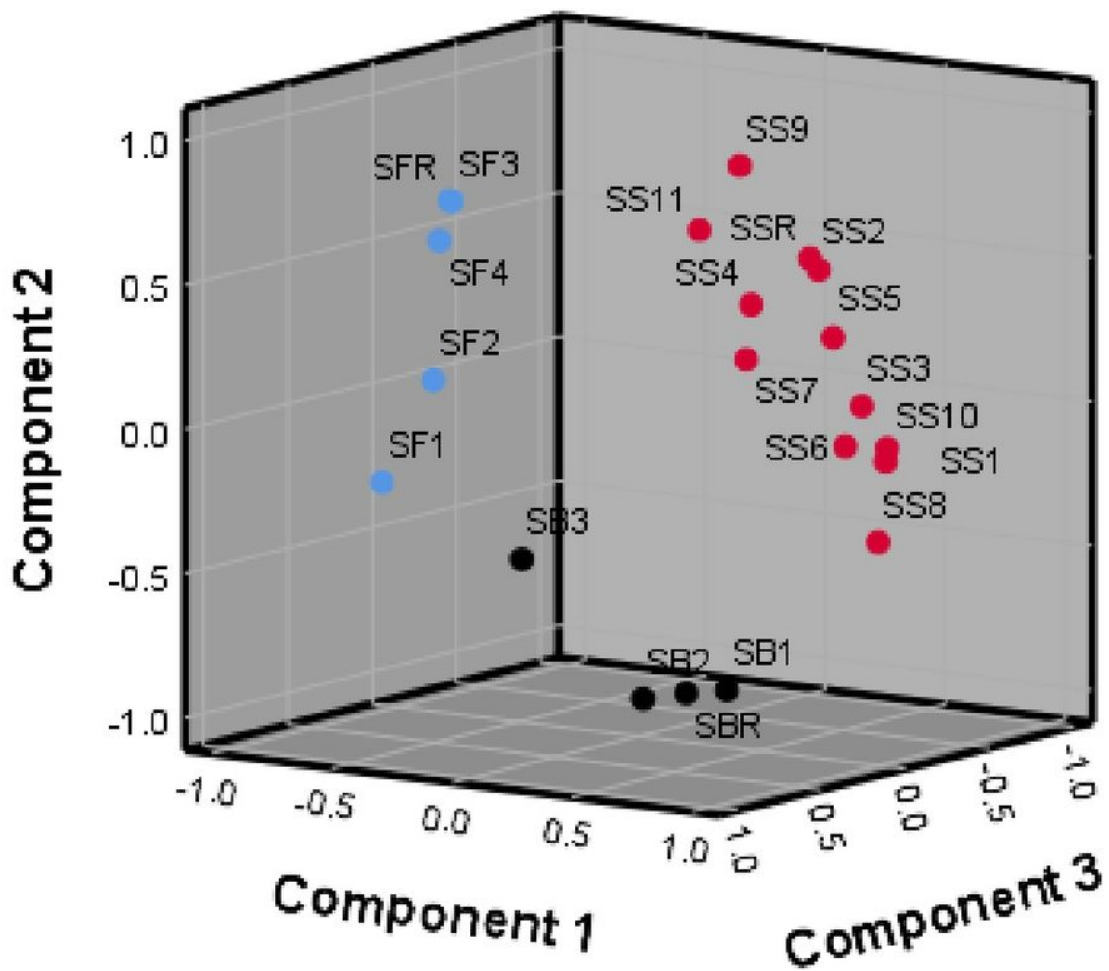


Figure 3

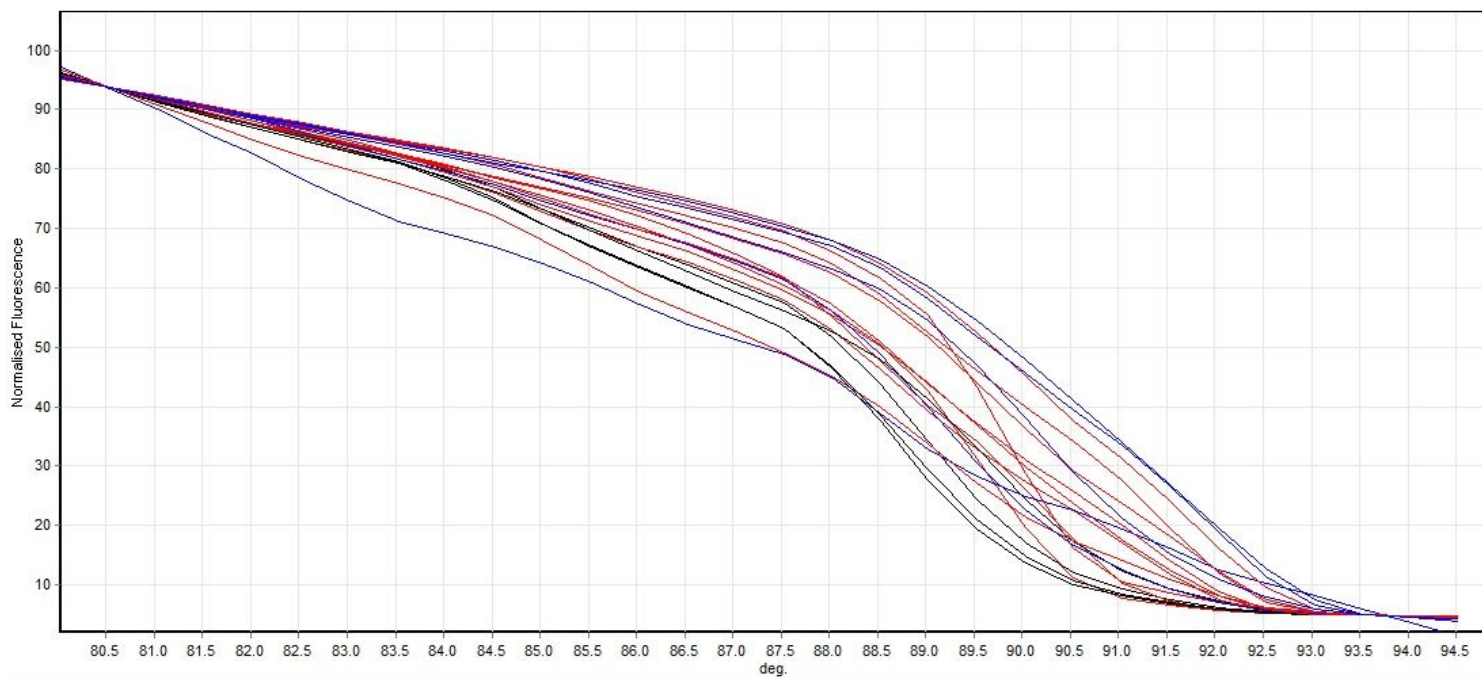


Figure 4

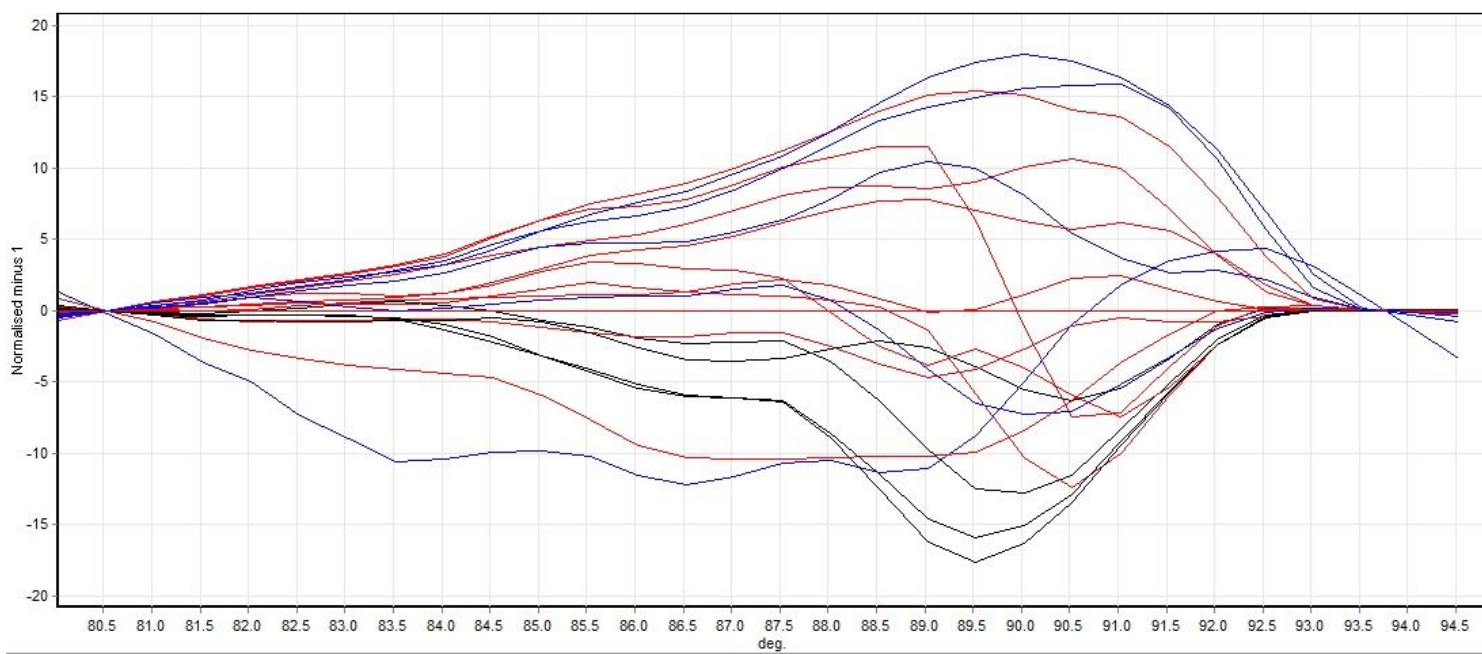


Figure 5

Component Plot

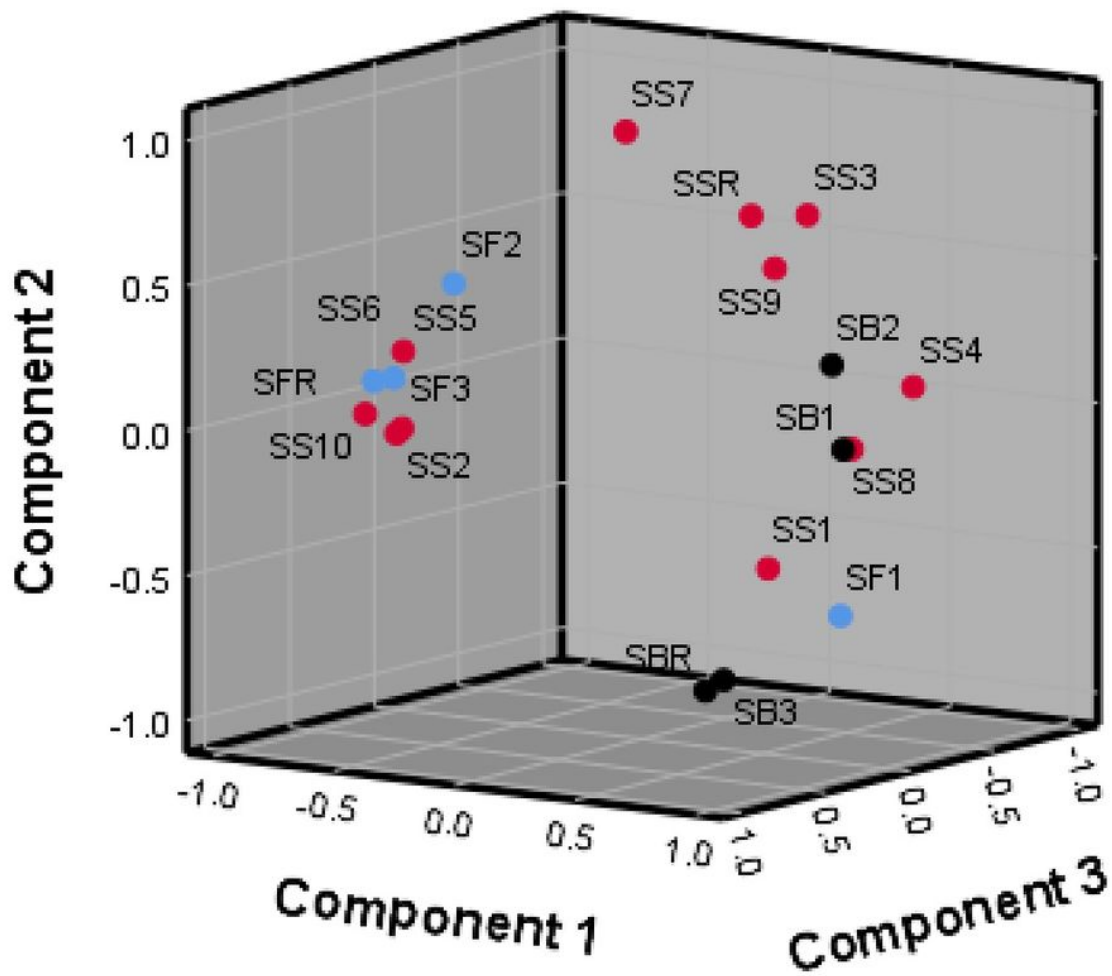


Figure 6

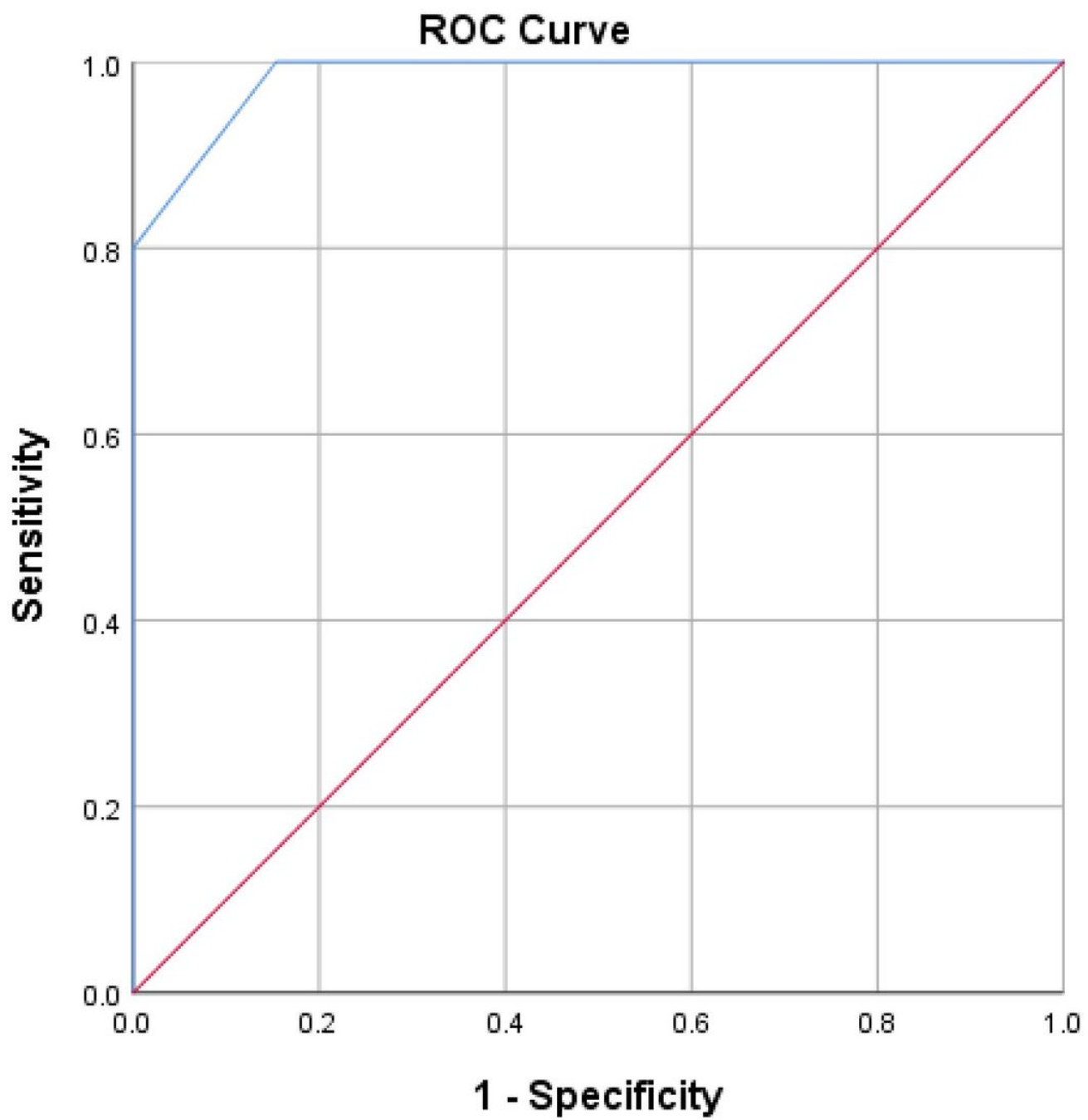


Figure 7

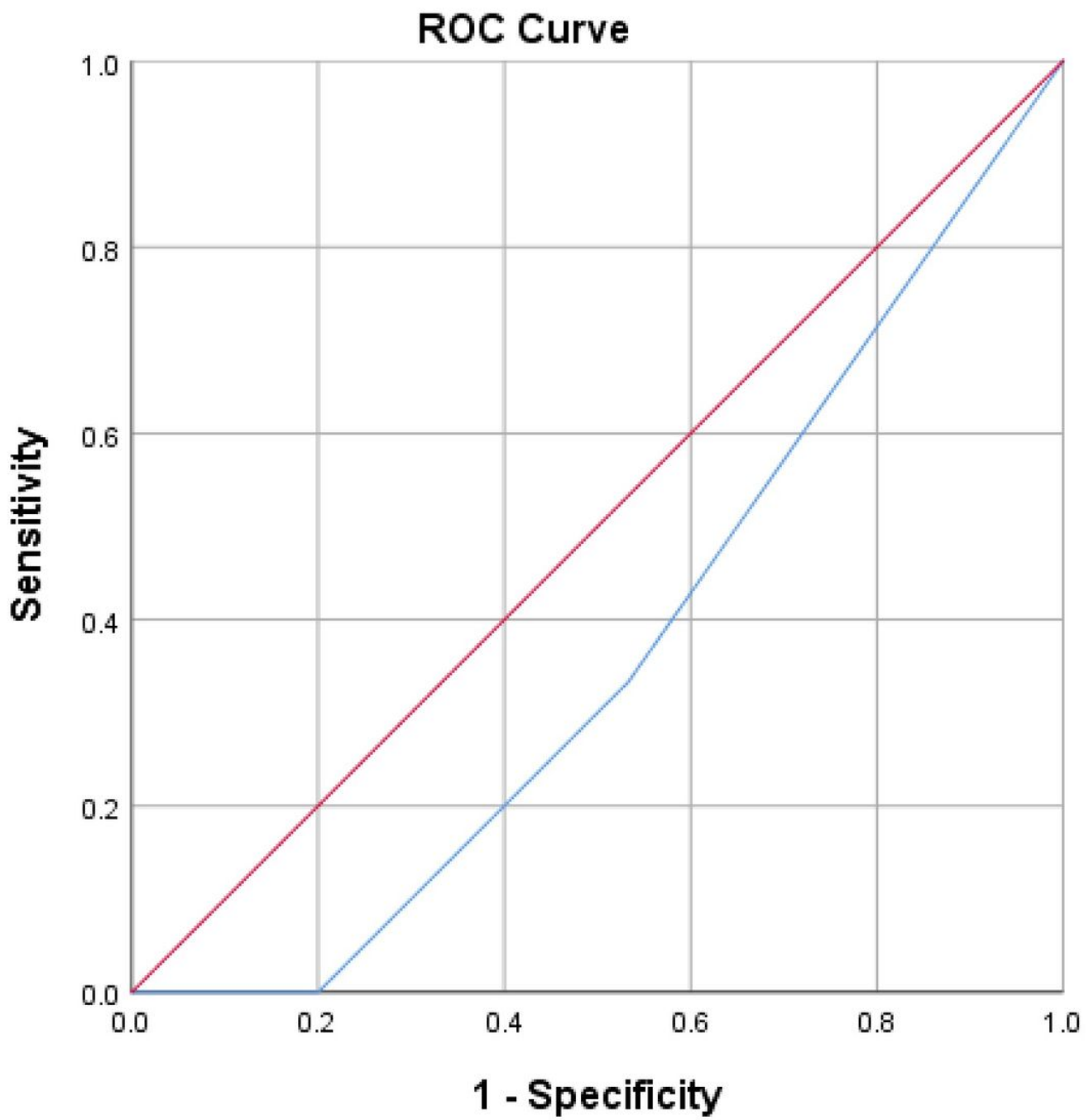


Figure 8