**Abstract. – Background and Objectives:** *Streptococcus pyogenes* (*S. pyogenes*) is an important cause of pharyngitis. Rapid detection of this microorganism in throat specimens is essential to promptly start antibiotic therapy which could be lead to prevent complications and stop transmission of infection to other individuals. In the present study, fluorescent in situ hybridization (FISH) was compared with culture method for the detection of *S. pyogenes* in throat swab specimens.

**Materials and Methods:** One hundred eleven patients with pharyngitis were included in this study. The throat swab specimens of these patients were investigated by both conventional culturing and FISH.

**Results:** Based on the results of this investigation, the sensitivity and specificity of FISH were 88.9% and 97.8%, respectively. Strikingly, in the specimen of one patient who had received antibiotic previous to clinical sampling, *S. pyogenes* was detected by means of FISH, whereas the culture method could not detect this bacterium.

**Conclusions:** It seems that FISH is a suitable method for quick identification of *S. pyogenes* in throat swab specimens. When FISH is positive, culturing is not necessary. But because of the limited sensitivity of FISH for detection of *S. pyogenes* in throat swab specimens, culturing should be performed if FISH was negative.

**Key Words:** *Streptococcus pyogenes*, Fluorescent in situ hybridization, FISH, Pharyngitis.

**Introduction**

*Streptococcus pyogenes* (*S. pyogenes*) is an important pathogen which causes both local and systemic infections. The most common infection due to this organism is streptococcal sore throat or pharyngitis. Suppurative sequelae of pharyngitis may ensue from spread of infectious agent to adjacent tissue or by bacteremia. Also, the poststreptococcal nonsuppurative disorder, rheumatic fever, sometimes develops after pharyngitis due to cross-reaction of streptococcal antigens with human heart tissue antigens. Therefore, rapid and reliable detection of *S. pyogenes* is essential for initiation of appropriate therapy, rapid eradication of the agent, and prevention of poststreptococcal disorders. Conventional bacteriological methods are relatively time-consuming so that require about 48 hours for isolation and identification of *S. pyogenes*. Moreover, antibiotic therapy before obtaining the specimens, may impede the isolation of the bacterium in culture media.

Fluorescent in situ hybridization (FISH) using fluorescently-labeled oligonucleotide probes that specifically target to ribosomal RNA (rRNA) is considered in microbiology as a molecular method for the rapid identification of bacteria. FISH can be used for the direct detection and visualization of microorganisms in clinical samples without previous amplification or cultivation steps and this would lead to a considerable reduced time for specific detection of infectious agents. There are many reports concerning the application of FISH for the detection of bacteria in clinical samples. For example, FISH has been used for diagnosis of *Helicobacter pylori* in gastric biopsy, *Enterococcus faecalis* and *Enterococcus faecium* in faeces, *Staphylococcus aureus* in sputum, oral streptococci in dental plaque, and several bacteria in cerebrospinal fluid (CSF) samples. Furthermore, *S. pyogenes*...
has been detected in samples such as maxillary sinus or fascial tissue by FISH. The objective of this study was to compare FISH with the conventional culture method for the detection of \textit{S. pyogenes} in throat swab specimens of patients suffering from pharyngitis.

**Materials and Methods**

**Collection and Cultivation of Throat Swab Specimens**

This study was approved by Ethical Committee of Bushehr University of Medical Sciences. Throat swab specimens were taken from 111 patients with pharyngitis. Two throat swabs were obtained from each patient. One swab was used for culture method and the other one for the FISH procedure. The specimens were cultured on blood agar (Merck, Germany) so that after streaking the medium, stabbing the inoculating loop into the agar was performed in order to allow subsurface growth and occur the most reliable hemolytic reactions. The blood agar plates were then incubated at 35°C. After 24 hours, the plates were observed to check the grown colonies. Negative cultures were reincubated for an additional 24 hours. The suspicious colonies were subcultured and then the identification of \textit{S. pyogenes} was carried out by means of conventional laboratory methods.

**Fixation of Samples for FISH**

The swabs for FISH were put into phosphate buffered saline (PBS) and rotated adequately to suspend the specimens in PBS. The specimens in these suspensions were then fixed by the adding an equal volume of absolute ethanol (Merck, Germany).

**Fixation of Reference Strains**

In the FISH procedure, \textit{S. pyogenes} (ATCC 19615) and \textit{Enterococcus faecalis} (ATCC 29212) were used as positive control and negative control, respectively. The strains were grown in Luria-Bertani (LB) broth. Then, the bacterial cultures were centrifuged at 8000 rpm for 5 minutes at 4°C. The bacterial pellets were resuspended in PBS and recentrifuged (PBS washing). The pellets were suspended again in 500 µl PBS and fixed by the addition of the equal volume of absolute ethanol. Fixed bacterial strains were stored at –20°C.

**FISH**

Two oligonucleotide probes, Strpyo and EUB338, that synthesized and 5’-labeled by Metabion ( Martinsried, Germany), were used for our study. Probe Strpyo (5′-CTA A CA TGC GTT AG T CTC TC- 3’) that specifically binds and hybridizes to a 16S rRNA position of \textit{S. pyogenes}, was applied for specific identification of this microorganism. The 5’ end of Strpyo was labeled with fluorescent dye Cy3 which exhibits red signal. Probe EU B 338 with the sequence 5′-GCT GCC TCC CGT A G G A G T- 3’ that targets and hybridizes 16S rRNA of nearly all bacteria, was labeled with Flu (green signal) at the 5’ end.

The FISH procedure was carried out by spotting 10 µl of each fixed throat specimen or fixed control reference strain on glass slides. After air drying, the slides were put in ethanol series (50%, 80%, and absolute ethanol; 3 minutes each) for dehydration. Enzymatic treatment was done with 1 mg/ml lysozyme (Sigma, Steinheim, Germany) for 15 minutes. In the hybridization step, each specimen or bacterial control was covered with 10 µl of hybridization buffer (0.9M NaCl, 20 mM Tris-HCl [pH 8], 0.01% SDS, 20% formamide) containing 5 ng/µl of each probe EUB338-Fluo and Strpyo-Cy3 and then the slides were reincubated at 46ºC for 90 minutes in humid chambers. For stringent washing, the slides were incubated in washing buffer (20 mM Tris-HCl [pH 8], 0.01% SDS, 225 mM NaCl) for 15 minutes at 48ºC. Afterwards, DAPI (4’,6-diamidine-2′-phenylindole dihydrochloride) (Roche, Mannheim, Germany) with the concentration 1 µg/ml for 5 minutes was applied for nonspecifically staining the DNA. In the FISH procedure, negative controls were also used.

**Analysis of Test**

The sensitivity and specificity of FISH were calculated with the formulas: 
\[ \text{Sensitivity} = \frac{a}{a + c} \times 100 \]
\[ \text{Specificity} = \frac{d}{b + d} \times 100 \]

where \( a = \) true positive, \( b = \) false positive, \( c = \) false negative, and \( d = \) true negative.
Results

Table I shows the results of the examination of 110 throat swab specimens for the detection of *S. pyogenes* using conventional culturing and FISH. Sixteen of the specimens were *S. pyogenes* positive by conventional culture and FISH. *S. pyogenes* was identified by FISH due to its specific red signal under the relevant filter of microscope (Figure 1). In 90 throat samples, *S. pyogenes* was not detected according to the both methods. *S. pyogenes* was detected in two specimens by means of culturing, while these specimens were FISH negative for *S. pyogenes*. Also, two throat specimens were culture negative but FISH positive for this bacterium. When we repeated the FISH tests, the results were same. Analysis of test in this study showed that the sensitivity and specificity of FISH for the detection of *S. pyogenes* in throat swab specimens were 88.9% and 97.8%, respectively.

Strikingly, in the throat specimen of one patient who had received antibiotic before clinical sampling, *S. pyogenes* was detected by FISH, whereas the culture was negative for this microorganism. We did not include this specimen in total number of samples in calculation of sensitivity and specificity.

Discussion

Pharyngitis is a common disease of *S. pyogenes* which has both suppurative (e.g. septicemia) and nonsuppurative (e.g. acute rheumatic fever) complications\(^1,2\). On the other hand, some viral diseases such as infectious mononucleosis and adenovirus infection can exhibit clinical manifestations similar to *S. pyogenes* pharyngitis\(^1\). For these reasons, every effort must be made to rapidly identify the causative agent, correct treatment of patients and thus prevention of life-threatening complications. Isolation and identification of *S. pyogenes* by conventional culturing is not fast. Application of FISH as a rapid molecular technique to detect various bacteria within the clinical specimens has already been reported in many studies\(^3,8,10,11,13,19\). Therefore, we designed the present study to evaluate FISH for detection of *S. pyogenes* in throat swab specimens.

Eighteen specimens were culture positive, of which 16 specimens were FISH positive, but 2 specimens were FISH negative (Table I). The sensitivity of FISH for detection of *S. pyogenes* was 88.9%. It is probable that the low number of *S. pyogenes* cells within 2 aforementioned specimens, and thus, microscopic detection limit of FISH could had been the reason of false negative results. Low number of bacterial cells in some specimens is a limitation factor for FISH which was also mentioned in other studies for samples such as sputum\(^3,11\) or gastric biopsy\(^8\).

By conventional culture, 92 specimens were culture negative for *S. pyogenes*, of which 90

Table I. Results of the examination of 110 throat swab specimens by conventional culturing and FISH for diagnosis of *Streptococcus pyogenes*.

<table>
<thead>
<tr>
<th>Number of throat specimens</th>
<th>Culture results</th>
<th>FISH results</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>90</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>Positive</td>
</tr>
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</table>
specimens were also FISH negative, whereas 2 specimens were FISH positive. The specificity of FISH was 97.8%. Although we have supposed the FISH results of these 2 specimens as false positive, but following explanations indicate that they might be the false negative results of culture. As first explanation, it has been shown that *S. pyogenes* has the capability to enter a quiescent state and formation of viable but non-culturable bacterial cells. On the other hand, during starvation the number of culturable *S. pyogenes* reduced quickly. However, bacterial number observed by FISH does not significantly change. Thus, perhaps the 2 mentioned throat samples in our study contained non-culturable *S. pyogenes* which did not grow in culture medium, but was viable and could be detected by FISH. A similar condition has been found in *H. pylori* that develops from vegetative form into non-cultivable form. The non-cultivable *H. pylori* possess an enough quantity of rRNA, so could be detected by means of FISH. The second explanation is that the normal bacterial flora of the mouth may obscure or suppress the growth of *S. pyogenes* in throat specimens. The third explanation is related to probe Strpyo. In the study performed by Trebesius et al, probe Strpyo was examined by hybridizing several different target and non-target species. The results of mentioned study have been shown that Strpyo is highly specific for *S. pyogenes*. Also, during another study, evaluation of the probe Strpyo was performed on pure cultures of clinical isolates as well as reference strains that Strpyo proved to be 100% sensitive and specific. Even if the FISH results of the 2 mentioned specimens in our investigation considered as false positive, the specificity of FISH is still high.

Since, the specificity of FISH for detection of *S. pyogenes* in throat was found to be higher than its sensitivity, in practice two throat swabs from each patient can be used. First, the FISH procedure can be performed for one throat swab specimen and if it was FISH-positive, the follow up of the other swab by culturing is not necessary.

In conclusion, it seems that FISH is relatively an appropriate technique for rapid detection of *S. pyogenes* in throat swab specimens. Because of the limited sensitivity of FISH, we should state that it only partially can be used instead of culture method. However, since a high specificity was achieved for FISH, positive results are reliable, so that antibiotic therapy can start without delay.

Acknowledgements

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