International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (6), pp. 001-005, June, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Evaluation of fluorescent *in situ* hybridization for rapid diagnosis of enterococcal wound infection

Somayyeh Gharibi[1], Saeed Tajbakhsh[1]*, Keivan Zandi[1] and Ramin Yaghobi[2]

[1]Department of Microbiology and Parasitology, Faculty of Medicine, The Persian Gulf Tropical and Infectious Diseases Research Center, Bushehr University of Medical Sciences, P. O. Box 3631, Bushehr, Iran. [2]Shiraz Transplant Research Center, Namazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.

Accepted 24 March, 2018

Enterococci are among prominent causes of nosocomial wound infections. Since the rapid detection of causative agents could make earlier administration of choice antibiotics and quick recovery of patients, so the application of rapid diagnostic methods is important. Therefore, this study was designed to evaluate fluorescent *in situ* hybridization (FISH) for the detection of *Enterococcus* in wound swab samples. The time needed for FISH procedure is about 3 h. Specimens taken from 33 hospitalized patients were examined by both FISH and culturing procedures. By using conventional culture, 10 of 33 wound samples were culture-positive. Out of these 10 specimens, eight were FISH-positive, but two specimens were FISH-negative for *Enterococcus.* The remaining 23 wound specimens were *Enterococcus* negative according to the both methods. Therefore, the specificity of FISH was 100%; however, this method showed 80% sensitivity. Because of high specificity of FISH, the combined application of FISH and cultivation methods would be suggested for detection of enterococci from wound specimens in situations in which rapid diagnosis has an advantage in the treatment of patients.

Key words: *Enterococcus*, wound infection, fluorescent *in situ* hybridization, FISH.

INTRODUCTION

Enterococci are gram-positive cocci that typically arranged in pairs and short chains. They are facultative anaerobes and are part of the normal human faecal flora (Sood et al., 2008). The majority of human infections are caused by *Enterococcus faecalis* and *Enterococcus faecium*, while other species are responsible for the less than 5% of infections (Cetinkaya et al., 2000; Brooks and Carroll, 2007; Sood et al., 2008). Before 1990s, enterococci have been recognized as the major cause of endocarditis (Ctinkaya et al., 2000; Sood et al., 2008). In the past decade, prevalence of enterococci has been increased in hospitals. Moreover, the spectrum of enterococcal infections has been changed (Sood et al., 2008). These organisms are important causes of nosocomial infections, particularly in intensive care units (Brooks and Carroll, 2007). Enterococci are intrinsically resistant to several commonly used antibiotics (Cetinkaya et al., 2000; Brooks and Carroll, 2007). This intrinsic resistance and also development of resistance to the other available antibiotics are major reasons for survival of enterococci in hospitals (Cetinkaya et al., 2000).

Among the common sites of enterocaccal colonization in the hospitalized patients are ulcers and soft tissue wounds (Sood et al., 2008). The enterococci have been reported as the second most frequent organisms that isolated from nosocomial wound infections (Winn et al., 2006; Sood et al., 2008). Detection of *Enterococcus* in wound specimens using conventional cultural methods is relatively time-consuming and requires 48 to 72 h (Ke et al., 1999; Cupakova et al., 2005; Waar et al., 2005; Winn et al., 2006; Brooks and Carroll, 2007). Therefore, rapid and reliable methods are useful for detection of *Enterococcus* in diagnostic laboratories. Fluorescent *in situ* hybridization (FISH) is a helpful molecular technique

*Corresponding author. E-mail: tajbakhshsaeed@yahoo.com, tajbakhsh@bpums.ac.ir. Fax: +98-771-2524044. Tel: +98-917-774 6164.

Abbreviations: FISH, Fluorescent *in situ* hybridization; **rRNA,** ribosomal RNA; **PNA,** peptide nucleic acid.

for rapid and specific detection of microorganisms in clinical samples (Trebesius et al., 2000; Hogardt et al., 2000; Tajbakhsh et al., 2008a), especially in mixed infections. Fluorescently-labeled probes are used in the FISH technique to specifically hybridize the ribosomal RNA (rRNA) and visualize the whole microbial cells via fluorescence microscopy (Trebesius et al., 2000). Thus, FISH technique permits specific detection and observation of microorganisms with an intact morphology within their natural environment or clinical specimens. FISH procedure takes only about 3 h. The purpose of our work was to evaluate the sensitivity and specificity of FISH for detection of genus *Enterococcus* in wound swab specimens of patients with wound infection. In this study, FISH was compared with conventional cultural method.

MATERIALS AND METHODS

This study was done from March 2009 to October 2009.

Wound swab specimens

Collection of wound specimens was approved by ethical committee of Bushehr University of Medical Sciences. Thirty-three hospitalized patients with wound infection were included in this study. The specimens from each patient were taken simultaneously by two sterile swabs. One swab was used for FISH method and the other swab used for cultural procedure.

Fixation of specimens and bacterial reference strains

The swabs for FISH were placed into phosphate buffered saline (PBS), then pressed and rotated adequately in order to release and suspend the specimens. Afterward, an equal volume of absolute ethanol (Merck, Darmstadt, Germany) was added to each suspended specimen. The fixed specimens were then ready for the FISH procedure. The control strains in this investigation were *E. faecalis* (ATCC, 29212) and *Streptococcus pyogenes* (ATCC, 19615). Each strain was grown and fixed for FISH as follows: the strain was cultured in Luria- Bertani broth (Hogardt et al., 2000; Kempf et al., 2000; Tajbakhsh et al., 2004) and then harvested by centrifugation at 8000 rpm for 5 min at 4°C. The supernatant was discarded and the bacterial pellet was washed with PBS. The bacterial cells were resuspended in PBS and fixed by an equal volume of absolute ethanol (Trebesius et al., 2000; Tajbakhsh et al., 2004).

The FISH method

Oligonucleotide probes EUB338 and Enc which were synthesized and 5'-labeled by Metabion (Martinsried, Germany) were used for the present study. Probe EUB338 (5'-GCT GCC TCC CGT AGG AGT- 3') that hybridizes almost all bacteria (Amann et al., 1990) was 5'-labeled with fluorochrome Fluo, the dye which emitted green color. Probe Enc (5'-CCC TCT GAT GGG TAG GTT-3') that targets and hybridizes *Enterococcus* spp, was used for detection of this bacterial genus (Kempf et al., 2000). The 5' end of probe Enc was labeled with fluorochrome Cy3 which is a cyanine dye with red fluorescent signal.

For FISH, 10 µl of fixed specimens or fixed control strains were placed onto microscopic slides and air-dried. In next step, the slides were submerged into 50, 80%, and absolute ethanol, respectively (Hogardt et al., 2000; Kempf et al., 2000; Trebesius et al., 2000; Tajbakhsh et al., 2004). In order to break peptidoglycan layer and permeabilization of bacterial cells, treatment with lysozyme (1 mg/ml in 10 mM Tris-HCl pH 8) (Sigma, Steinheim, Germany) was performed (Trebesius et al., 2000). This partial cell wall digestion was stopped after 15 min by washing the glass slides with PBS and immersing them in absolute ethanol and followed by air drying. Then, a mixture of two probes EUB338 and Enc was prepared in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% SDS, 20% formamide).The concentration of each probe in hybridization buffer was 5 ng/µl. Hybridization step was carried out by adding 10 µl of the mentioned hybridization buffer containing probes to the each specimen or bacterial control strain and incubating the slides at 46°C for 90 min in a humid condition. Afterward, the unbound probes were removed by gently rinsing the slides with several milliliters of prewarmed washing buffer (20 mM Tris-HCl [pH 8], 0.01% SDS, 225 mM NaCl) followed by a 15 min immersing the slides in washing buffer at 48°C (Trebesius et al., 2000; Tajbakhsh et al., 2004).

Staining of DNA was done with 1 µg/ml 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany) for 5 min (Trebesius et al., 2000; Russmann et al., 2001; Samarbaf-Zadeh et al., 2006; Gescher et al., 2008; Tajbakhsh et al., 2008b). One drop of fluorescent mounting medium (DAKO, Glostrup, Denmark) was dispensed on glass slides and a coverslip was applied over mounting medium on each slide. Subsequently, we examined the slides with an epifluorescence microscope (Nikon 80i, Tokyo, Japan) and took the pictures by means a DS-5Mc-L1 digital camera (Nikon, Tokyo, Japan) which was an equipment of aforementioned microscope.

Culturing of *Enterococcus* from wound specimens

The swabs for culture procedure were inoculated on to blood agar plates and bile esculin agar which were then incubated at 35°C for 24 h (Winn et al., 2006; Forbes et al., 2007). Suspicious colonies which represented gram positive cocci by gram staining were sub-cultured for purity. Grown colonies in pure cultures were examined with the conventional tests (Larsen, 2000; Winn et al., 2006; Forbes et al., 2007).

RESULTS

Detection of *Enterococcus* by FISH technique using probes EUB338-Fluo and Enc-Cy3 as well as staining with DAPI is shown in Figure 1. The results of the examinations of wound swab samples from 33 patients with wound infection are shown in Table 1. Eight specimens were *Enterococcus* positive according to culture and FISH methods (Group 1), whereas 23 specimens were negative for *Enterococcus* by both methods (Group 2). In two specimens, *Enterococcus* was detected by culturing, but not by FISH (Group 3); we found an overgrowth of *Pseudomonas* sp. in the culture of one of the two mentioned specimens and *Proteus* sp. in the culture of the other one. All FISH-positive samples were culture-positive, that is, FISH did not produce any false positive result. Therefore, the specificity of FISH was 100%. Based on the study, the sensitivity of FISH for detection of *Enterococcus* in wound swab specimens was 80%, since two false-negative results were found.

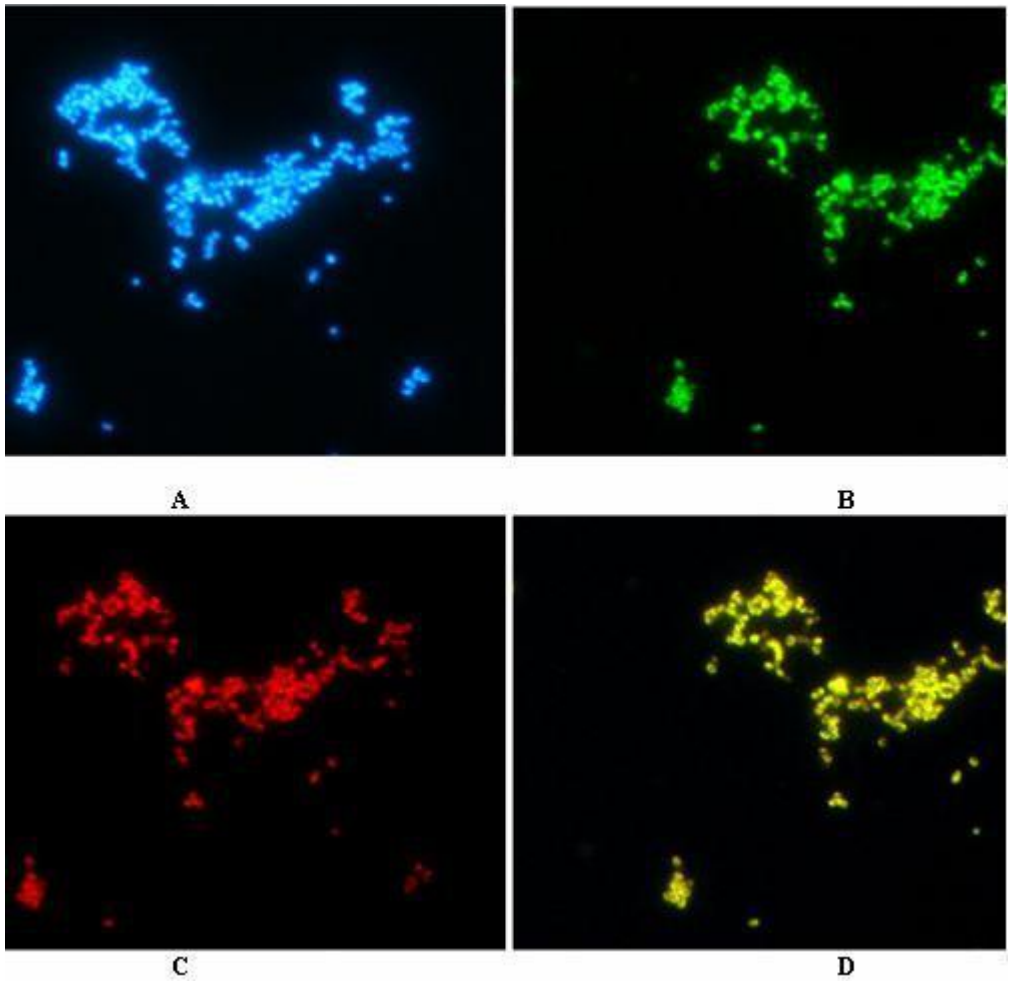

Figure 1. Detection of *Enterococcus* by FISH. All of four panels show the identical microscopic field using relevant filter. Panel A demonstrates cocci with the blue fluorescence because staining of DNA with DAPI. Panel B shows cocci in green, indicating hybridization with probe EUB338-Fluo. Panel C indicates hybridization with probe Enc-Cy3 because the cocci exhibit red fluorescence signal. In panel D, mixture of green and red colors produces a yellow signal.

Table 1. Thirty-three wound swab specimens tested by culture and FISH methods for detection of genus *Enterococcus*.

Group	Number of specimens	Results	
		Culture	FISH
1	8	Positive	Positive
2	23	Negative	Negative
3	2	Positive	Negative

DISCUSSION

Enterococci are prominent causes of nosocomial infections world- wide and are associated with a high mortality (Mathur et al., 2003). They are important causes of wound infection (Sood et al., 2008). Rapid diagnosis of enterococcal infections could lead to rapid administration of choice antibiotics, rapid recovery of patients, makes shorter hospitalization, and reduce the patient's expenses (Gescher et al., 2008) . Rapid detection is also important in reducing the spread of multiresistant enterococci (Ke et al., 1999). Thus, we evaluated FISH for detection of *Enterococcus* in wound swab specimens and get a high specificity (100%). However, sensitivity of FISH was 80%.

As shown in Table 1 and Group 3, two culture-positive specimens were FISH-negative. These false-negative results may be due to low number of enterococcal cells within two mentioned specimens. Overgrowth of *Pseudomonas* sp. in one of the two mentioned specimens and *Proteus* sp. in the other one probably reduced the growth of *Enterococcus* sp. and this phenomenon made some difficulty for us during isolation of *Enterococcus* form these specimens. Low number of enterococcal cells as compared with the number of *Pseudomonas* or *Proteus* cells could have been the

reason of reducing the growth and appear a few colonies of enterococci in the culture of these two specimens and also negative results of FISH. In fact, low number of microbial cells within some specimens causes a microscopic detection limit of FISH. This limitation has also been reported in other investigations e.g. for bacteria such as *Staphylococcus aureus* in sputum samples (Hogardt et al., 2000; Tajbakhsh et al., 2004) or *Helicobacter pylori* in gastric tissues (Rüssmann et al., 2001; Samarbaf-Zadeh et al., 2006).

By conventional culture, 23 wound samples were culture-negative for *Enterococcus*. These 23 samples were also FISH-negative that demonstrates a high specificity of FISH. With attention to achieved sensitivity and specificity for FISH in this study, we suggest sampling from wounds simultaneously by two swabs, one for FISH and the other one for culture. Because of the high specificity of FISH, the FISH-positive results are reliable, thus, enterococcal antibiotic therapy can start rapidly and finally after recognition of the results of culture and antibiotic susceptibility tests, previous administrated drugs can be replaced with more effective antibiotics if necessary. But, because of the limited sensitivity of FISH, cultivation methods should be completed for FISH-negative specimens before starting antibiotic therapy. Thus, the patients have a chance to be treated 2 to 3 days earlier, since FISH procedure takes only about 3 h.

We used oligonucleotide probes in our study.

Also, some other researchers used oligonucleotide probes for detection of *Enterococcus* species in faeces and /or blood cultures (Waar et al., 2005; Wellinghausen et al., 2007). Nowadays, peptide nucleic acid (PNA) probes are also accessible to detect enterococci (Forrest et al., 2008). Malic and colleagues utilized PNA-FISH to detect biofilm-forming bacteria including *Pseudomonas aeruginosa* and *S. aureus* in human chronic skin wounds and reported this technique as a reliable method for study of biofilms (Malic et al., 2009). PNA probes have a higher affinity for complementary sequence than conventional oligonucleotide probes (Amann and Fuchs, 2008), however preparation of PNA probes is demanding and expensive (Schweickert et al., 2004; Amann and Fuchs, 2008).

In conclusion, although FISH showed 80% sensitivity, but because of high specificity and reliable positive results, we suggest the application of FISH to detect enterococci from wound swab specimens in situations in which rapid diagnosis has an advantage in the therapy of the patients.

ACKNOWLEDGEMENTS

The authors wish to thank the Vice-chancellor of Research of Bushehr University of Medical Sciences for financial support. They also thank Mr. Omid Gharibi for his guidelines in the culture and isolation of *Enterococcus*.

REFERENCES

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol., 56: 1919-1925.

Amann R, Fuchs BM (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol., 6: 339-348.

Brooks GF, Carroll KC (2007). The streptococci. In: Brooks GF, Carroll KC, Butel JS, Morse SA (eds). Jawetz, Melnick and Adelberg's Medical Miicrobiology. 24th ed. Mc Grow Hill, New York, pp. 233-248.

Cetinkaya Y, Falk P, Mayhall CG (2000). Vancomycin-resistant enterococci. Clin. Microbiol. Rev., 13: 686-707.

Cupakova S, Pospisilova M, Kolackova I, karpiskova R (2005). Genus-specific identification of enterococci by PCR method. Acta. Vet. Brno., 74: 633-637.

Forbes BA, Sahm DF, Weissfeld AS (2007). *Streptococcus, Enterococcus* and similar organisms. In: Bailey and Scott's Diagnostic Microbiology. 12th ed. Mosby Elsevier, St. Louis, pp. 265-280.

Forrest GN, Roghmann MC, Toombs LS, Johnson JK, Weekes E, Lincalis DP, Venezia RA (2008). Peptide nucleic acid fluorescent *in situ* hybridization for hospital-acquired enterococcal bacteremia: delivering earlier effective antimicrobial therapy. Antimicrob. Agents Chemother., 52: 3558-3563.

Gescher DM, Kovacevic D, Schmiedel D, Siemoneit S, Mallmann C, Halle E, Göbel UB, Moter A (2008). Fluorescence *in situ* hybridisation (FISH) accelerates identification of Gram-positive cocci in positive blood cultures. Int. J. Antimicrob. Agents., 325: 551-559.

Hogardt M, Trebesius K, Geiger AM, Hornef M, Rosenecker J, Heesemann J (2000). Specific and rapid detection by fluorescent *in situ* hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. J. Clin. Microbiol., 38: 818-825.

Ke D, Picard FJ, Martineau F, Ménard C, Roy PH, Ouellette M, Bergeron MG (1999). Development of a PCR assay for rapid detection of enterococci. J. Clin. Microbiol., 37: 3497-3503.

Kempf VAJ, Trebesius K, Autenrieth IB (2000). Fluorescent *in situ* hybridization allows rapid identification of microorganisms in blood cultures. J. Clin. Microbiol., 38: 830-838.

Larsen HS (2000). *Streptococcaceae*. In: Mahon CR, Manuselis G (eds).Text Book of Diagnostic Microbiology. 2nd ed. W.B. Saunders Company, Philadelphia, pp. 346-371.

Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW (2009). Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent *in situ* hybridization (PNA FISH). Microbiology., 155: 2603-2611.

Mathur P, Kapil A, Chandra R, Sharma P, Das B (2003). Antimicrobial resistance in *Enterococcus faecalis* at tertiary care centre of northern India. Indian. J. Med. Res., 118: 25-28.

Rüssmann H, Kempf VAJ, Koletzko S, Heesemann J, Autenrieth IB (2001). Comparison of fluorescent *in situ* hybridization and conventional culturing for detection of *Helicobacter pylori* in gastric biopsy specimens. J. Clin. Microbiol., 39: 304-308.

Samarbaf-Zadeh AR, Tajbakhsh S, Moosavian SM, Sadeghi-Zadeh M, Azmi M, Hashemi J, Masjedi-Zadeh A (2006). Application of fluorescent *in situ* hybridization (FISH) for the detection of *Helicobacter pylori.* Med. Sci. Monit., 12: CR426-430.

Schweickert B, Moter A, Lefmann M, Göbel UB (2004). Let them fly or light them up: matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry and fluorescence *in situ* hybridization (FISH). APMIS., 112: 856-885.

Sood S, Malhotra M, Das BK, Kapil A (2008). Enterococcal infections & antimicrobial resistance. Indian J. Med. Res., 128: 111-121.

Tajbakhsh S, Moosavian M, Samarbaf-Zadeh AR, Sadeghi-Zadeh M, Adler K, Hogardt M, Heesemann J (2004). Rapid detection of *Staphylococcus aureus* and genus *Staphylococcus* by fluorescent *in situ* hybridization (FISH). Iranian South Med. J., 6: 104-114.

Tajbakhsh S, Hogardt M, Heesemann J, Grzonka C, Adler K (2008a). Detection of *Pseudomonas aeruginosa* in sputum samples by modified fluorescent *in situ* hybridization. Afr. J. Biotechnol., 7: 553-556.

Tajbakhsh S, Samarbaf-Zadeh AR, Moosavian M (2008b). Comparison of fluorescent *in situ* hybridization and histological method for the diagnosis of *Helicobacter pylori* in gastric biopsy samples. Med. Sci. Monit., 14: BR183-187.

Trebesius K, Leitritz L, Adler K, Schubert S, Autenrieth IB, Heesemann J (2000). Culture independent and rapid identification of bacterial pathogens in necrotising fasciitis and streptococcal toxic shock syndrome by fluorescence *in situ* hybridisation. Med. Microbiol. Immunol., 188: 169-175.

Waar K, Degener JE, van Luyn MJ, Harmsen HJM (2005). Fluorescent *in situ* hybridization with specific DNA probes offers adequate detection of *Enterococcus faecalis* and *Enterococcus faecium* in clinical samples. J. Med. Microbiol., 54: 937-944.

Wellinghausen N, Bartel M, Essig A, Poppert S (2007). Rapid identification of clinically relevant *Enterococcus* species by fluorescence *in situ* hybridization. J. Clin. Microbiol., 45: 3424-3426.

Winn Jr WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC, Woods GL (2006). Gram-positive cocci PartII: streptococci, enterococci, and the "*Streptococcus*-like" bacteria. In: Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 672-764.