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Enumeration of *Legionella* CFU by Colony Hybridization Using Specific DNA Probes

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Oligonucleotide probes and colony hybridization (CH) were applied to enumerate organisms of the genus *Legionella* in cooling tower water. The CH counts indicated almost the same results as CFU counts in cultivated samples derived from the water. It was concluded that it is possible to substitute the CH procedure for the conventional one.

Legionella is the etiologic agent of Legionnaires' disease. The combination of isolation on selective medium and identification by DNA probe is useful in attempting to detect proliferating *Legionella* organisms (1, 2). Here we report two kinds of oligonucleotide probes, one targeting 16S rRNA and the other targeting a new target region, namely, an antigen common to the genus *Legionella*. Subsequently, we applied these probes to enumerate viable legionellae in various cooling tower water samples by using colony hybridization (CH). As a result of this investigation, we successfully identified and detected pinpoint *Legionella* colonies by using each of the probes.

Design and labeling of specific oligonucleotide probes for *Legionella*. The probes LEG225 (6), CA/G L253, CA/G L301 (3, 4, 8), and L5S-1 (5), together with their sequences and target positions, are listed in Table 1. The probe L5S-1 was used as a positive control.

The synthesized oligonucleotides were labeled with a digoxigenin oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany), in accordance with the manual.

Bacterial strains. The bacterial strains used in this study, along with their sources, are listed in Tables 2 and 3. *Legionella* strains were obtained from the stock strains of the Department of Microbiology, Gifu University School of Medicine. The reference strains were supplied by the Department of Bacteriology, Juntendo University.

Cell lysis for DNA extraction and purification. *Legionella* strains were cultivated at 37°C for 3 to 5 days on a 0.1% (wt/vol) α-ketoglutarate (BCYEα) agar plate (Eiken Chemical Co., Tokyo, Japan). About 10^8 to 10^9 cells were supplied in 533 μl of 10 mM Tris-HCl–1 mM EDTA buffer (pH 8.0). Sixty μl of 10% sodium dodecyl sulfate (SDS) (final concentration, 1%) and 6 μl of 10-mg/ml proteinase K solution (final concentration, 100 μg/ml) were added. After the solutions were mixed, they were incubated for 1 h at 37°C.

Reference microorganisms except *Staphylococcus aureus* were cultured in Luria-Bertani broth overnight and harvested. About 10^8 to 10^9 cells were suspended in 2 ml of 10 mM Tris-HCl–1 mM EDTA buffer (pH 8.0). One hundred μl of 10% SDS (final concentration, 0.5%) and 20 μl of 10-mg/ml proteinase K solution (final concentration, 100 μg/ml) were added. After the solutions were mixed, the mixture was incubated for 1 h at 37°C. Then 353 μl of 5 M NaCl and 282 μl of cetyltrimethylammonium bromide-NaCl were added, and the solution was incubated for 10 min at 65°C. *Staphylococcus aureus* was cultured overnight in Luria-Bertani broth and harvested. The cells were washed twice in 2 ml of 10 mM Tris-HCl–10 mM EDTA buffer (pH 8.0). The pellet was suspended in 2 ml of 10 mM Tris-HCl–10 mM EDTA buffer (pH 8.0) and then mixed sufficiently with 40 μl of achromopeptidase (50,000 U/ml in 10 mM NaCl). The solution was incubated at 55°C until the cells were lysed.

The subsequent DNA extraction and purification procedures were performed as described previously (7).

Detection of legionella colonies. The cultivation and identification of legionella colonies from the cooling tower water were performed as described previously (1). The replicated agar plates were reincubated for another 2 to 3 days at 37°C until the colonies grew to a visible size.

Testing of oligonucleotide probe specificity. One hundred ng of nucleic acid was blotted onto a 0.22-μm-pore-size Biodyne A nylon membrane (Poll Europe Ltd.). Subsequent dot blot hybridization was performed under the standard 5× SSC pro-

TABLE 1. Probe sequences and target positions

Probe	Sequence	Target position
LEG225	5′-AAgATTAgCCTgCgTCCgAT-3′	16S rRNA
CA/G L253	5′-ggTgATggTACTACTACTgC-3′	Common antigen
CA/G L301	5′-gAAggTCACAAAgCAgTTgC-3′	Common antigen
L5S (16)	5′-CTCgAACTCAgAAgTCAAACA TTTCCgCgCCAATgATAgTgTgA ggCTTC-3′	5S rRNA

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TABLE 2. *Legionella* strains used for probe specificity examinations and dot blot hybridization results

Legionella organism	Origin[a]	Reaction with probe at indicated concn [pmol/ml][b]				
		LEG225 [0.3]	CA/G L253 [1]	CA/G L301 [1]	CA/G L253 and CA/G L301 [1]	L5S-1 [0.3]
L. jordanis	GIFU 3193^T	++	+	+	++	++
L. pneumophila	GIFU 9134^T	++	++	++	++	++
L. bozemanae	GIFU 9140^T	++	+	++	++	++
L. micdadei	GIFU 9141^T	++	++	++	++	++
L. gormanii	GIFU 9142^T	++	+	+	++	++
L. longbeachae	GIFU 9245^T	++	+	++	++	++
L. pneumophila subsp. *fraseri*	GIFU 9246^T	++	++	++	++	++
L. dumoffii	GIFU 9247^T	++	+	±	+	++
L. oakridgensis	GIFU 10061^T	++	+	±	++	++
L. wadsworthii	GIFU 10062^T	++	++	−	++	++
L. feeleii	GIFU 10063^T	++	+	+	++	++
L. sainthelensi	GIFU 10392^T	++	+	−	++	++
L. hackeliae	GIFU 10740^T	++	+	±	+	++
L. jamestowniensis	GIFU 10741^T	++	++	++	++	++
L. cherrii	GIFU 10742^T	++	++	+	++	++
L. rubrilucens	GIFU 10743^T	++	−	−	+	++
L. maceachernii	GIFU 10745^T	++	+	±	+	++
L. spiritensis	GIFU 11199^T	++	−	±	+	++
L. israelensis	GIFU 11367^T	±	−	+	++	++
L. parisiensis	GIFU 11745^T	++	±	+	++	++
L. santicrucis	GIFU 11746^T	++	±	−	+	++
L. steigerwaltii	GIFU 11747^T	++	++	++	++	++
L. erythra	GIFU 11748^T	++	±	−	+	++
L. birminghamensis	GIFU 11749^T	+	++	++	++	++
L. anisa	GIFU 12075^T	++	±	±	++	++
L. cincinnatiensis	GIFU 12201^T	++	++	±	++	++
L. quinlivanii[c]	GIFU 12648^T					
L. moravica	GIFU 12649^T	±	++	±	++	++
L. brunensis	GIFU 12655^T	++	−	++	++	++
L. tucsonensis	GIFU 12656^T	++	++	++	++	++
L. adelaidensis	GIFU 13562^T	++	+	++	++	++
L. fairfieldensis	GIFU 13563^T	++	+	±	++	++
L. gratiana	GIFU 13564^T	++	±	±	++	++
L. lansingensis	GIFU 13565^T	++	++	±	++	++
L. shakespearei	GIFU 13566^T	±	++	++	++	++
L. pneumophila subsp. *pascullei*	GIFU 13567^T	++	++	++	++	++
Total hybridization rate for *Legionella* spp. [%]		91	74	57	100	100

[a] GIFU, Gifu University School of Medicine.
[b] ++, Strong hybridization signal; +, moderately strong hybridization signal; ±, weak hybridization signal; −, no hybridization signal.
[c] DNA extraction failed.

TABLE 3. Reference strains used for probe specificity examinations and dot blot hybridization results

Organism	Origin[a]	Reaction with probe at indicated concn [pmol/ml][b]				
		LEG225 [0.3]	CA/G L253 [1]	CA/G L301 [1]	CA/G L253 and CA/G L301 [1]	L5S-1 [0.3]
Enterobacter cloacae	ATCC 23355^T	−	−	−	±	++
Escherichia coli	ATCC 25922^T	−	−	−	±	++
Klebsiella pneumoniae	ATCC 13883^T	−	++	±	++	++
Proteus vulgaris	ATCC 13315^T	−	−	±	±	++
Pseudomonas aeruginosa	ATCC 27853^T	−	−	−	±	++
Salmonella enterica serovar Typhimurium	ATCC 14028^T	−	−	−	±	++
Serratia marcescens	ATCC 8100^T	±	−	−	±	++
Staphylococcus aureus	ATCC 12600^T	−	±	−	±	++
Total hybridization rate [%]		0	12.5	0	12.5	100

[a] ATCC, American Type Culture Collection.
[b] ++, Strong hybridization signal; +, moderately strong hybridization signal; ±, weak hybridization signal; −, no hybridization signal.

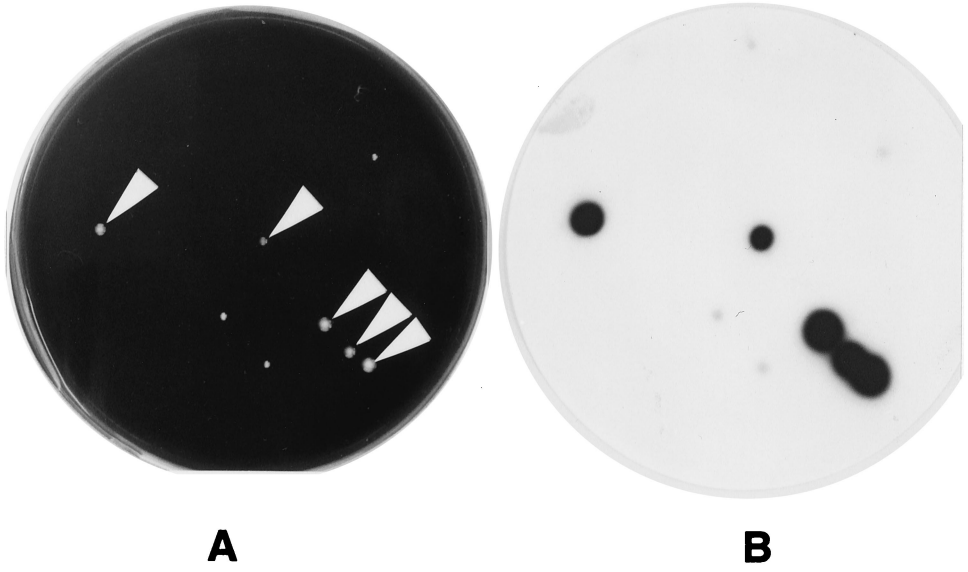

FIG. 1. Cultivated colonies of *Legionella* spp. from cooling tower water samples and CH results. The sample water was concentrated and mixed with 0.2 M KCl-HCl buffer (pH 2.2) for 4 min at 25°C. Then, 0.1 ml of mixture was streaked on WYOα agar. The plate was incubated at 37°C. (A) The plate after 4 days of incubation, just before the membrane was laid on the culture. The white arrows indicate *Legionella* colonies. (B) Signals of X-ray film developed after the plate shown in panel A was exposed to a membrane which was hybridized with a mixed probe consisting of probes CA/G L253 and CA/G L301.

cedure (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in a digoxigenin system (Boehringer Mannheim).

CH. The concentrated cooling tower water was cultured on a WYOα agar plate at 37°C for 2 to 5 days. Colonies were transferred from plates to 0.22-μm-pore-size (82-mm-diameter) Biodyne A nylon membranes (Poll Europe) by using procedures described previously (7). After replication, the membranes were placed colony-side-up on the paper (3MM; Whatman) and were saturated with 5% Triton X-100 in 10 mM Tris-HCl–1 mM EDTA (pH 8.0) for 1 h at 60°C. The membranes were transferred onto the paper (3MM); supplemented with 0.1 mg of proteinase K per ml in 10 mM Tris-HCl (pH 8.0) containing 10 mM NaCl, 1 mM sodium citrate, and 1.5% SDS; and incubated for 1 h at 60°C to lyse the cells. Subsequently,

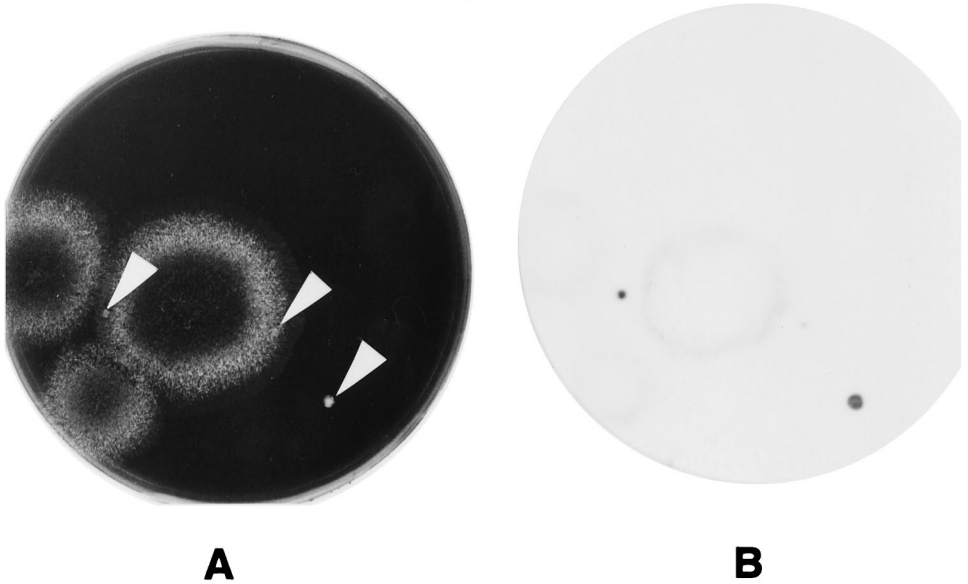

FIG. 2. Cultivated colonies of *Legionella* spp. from cooling tower water samples and CH results. The sample water was concentrated and mixed with 0.2 M KCl-HCl buffer (pH 2.2) for 4 min at 25°C. Then, 0.1 ml of mixture was streaked on WYOα agar. The plate was incubated at 37°C. (A) The plate after 4 days of incubation, just before the membrane was laid on the culture. The white arrows indicate *Legionella* colonies. (B) Signals of X-ray film developed after the plate shown in panel A was exposed to a membrane which was hybridized with a mixed probe consisting of probes CA/G L253 and CA/G L301.

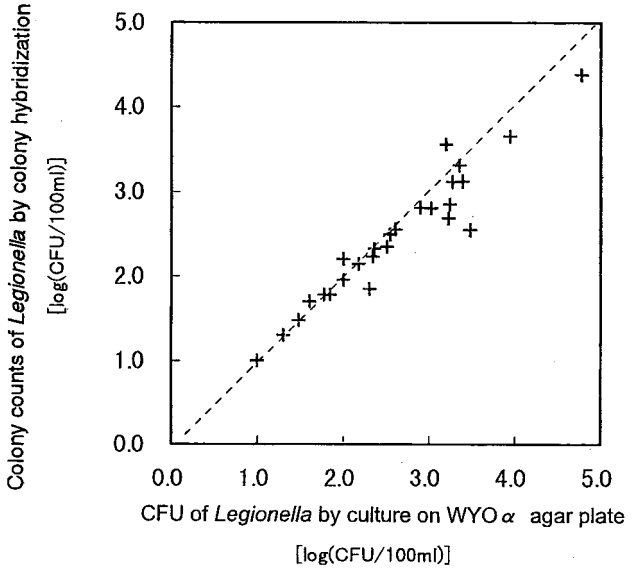

FIG. 3. Regression analysis of results of *Legionella* colony counts from samples of cooling tower water by CH with probe LEG225 against results of CFU counts by cultivation on WYOα agar plates. Regression coefficient, 0.96.

denaturing and neutralization of the DNA were performed as described previously (7). In order to remove the protein, the DNA fixed in the membranes was incubated overnight at 37°C with gentle agitation in a solution including 0.1 mg of proteinase K per ml, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% SDS. The membranes were washed twice for 5 min at room temperature with about 50 ml of 6× SSC per 100 cm^2.

Hybridization was carried out by using the same procedure described for dot blot hybridization, except that 10% blocking reagent was used.

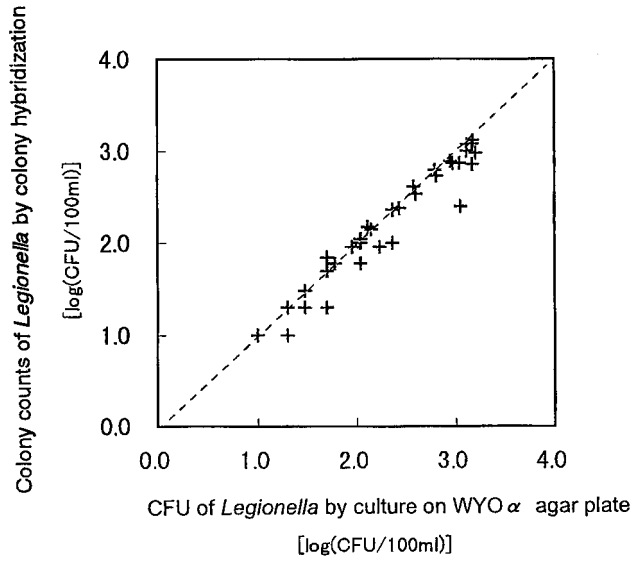

FIG. 4. Regression analysis of results of *Legionella* colony counts from samples of cooling tower water by CH with a mixed probe consisting of probes CA/G L253 and CA/G L301 against results of CFU counts by cultivation on WYOα agar plates. Regression coefficient, 0.98.

Specificity of the oligonucleotide probes. Probe LEG225 hybridized to 32 of the tested strains of *Legionella* spp. (91%) (Table 2). No false-positive hybridization occurred with the reference strain (Table 3). Probes CA/G L253 and CA/G L301 could hybridize 26 strains (74%) and 18 strains (54%) of the *Legionella* species tested, respectively. A mixed probe consisting of probes CA/G L253 and CA/G L301 together could hybridize 100% of the *Legionella species* tested (Table 2), but false-positive hybridization occurred with *Klebsiella pneumoniae* (Table 3).

Other bacteria and fungi which grew on the WYOα agar plates frequently disturbed the growth of legionellae and of the screened legionella colonies. However, as shown in Fig. 1, the signals from *Legionella* colonies clearly discriminated among these other microorganisms. A *Legionella* colony formed within a fungus colony was also detected (Fig. 2).

The results of CH counts of organisms from cooling tower water by using probe LEG225 and the results of CFU counts by cultivation revealed a close relationship, with a regression coefficient of 0.96 (Fig. 3).

In the case of the mixed probe consisting of probes CA/G L253 and CA/G L301 together, the results of CH counts were generally in agreement with those of CFU counts by plate culture; the regression coefficient was 0.98 (Fig. 4). The results of CH counts were slightly lower than those yielded by CFU counts of WYOα agar plate cultures. This tendency became remarkable in the samples that contained more than 100 CFU per 100 ml. This phenomenon is considered to result from the creation of large spots on the membrane by individual adjacent colonies because the colonies were aggregated after the process of colony transfer and lysing. But this phenomenon did not significantly affect the result of *Legionella* enumeration. The CH method has excellent features, including the ability to simultaneously perform enumeration of colonies and identification of every colony on a single plate.

Grimont et al. (2) reported that pinpoint *Legionella pneumophila* colonies were detectable by the use of a DNA probe labeled with a radioactive isotope after 4 days of incubation on BCYEα agar. In our study, we demonstrated the ability to enumerate *Legionella* colonies by DNA probe without radioactive-isotope labeling after the samples were cultured for 3 days.

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