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Specific and quantitative detection of PCR products from *Clostridium piliforme*, *Helicobacter bilis*, *H. hepaticus*, and mouse hepatitis virus infected mouse samples using a newly developed electrochemical DNA chip

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Abstract

We developed a microfabricated electrochemical DNA chip for detection of polymerase chain reaction (PCR) products from 16S rRNA sequences of *Clostridium piliforme* (Cp), *Helicobacter bilis* (Hb) and *Helicobacter hepaticus* (Hh), and the nucleocapsid protein gene of mouse hepatitis virus (MHV). This chip does not require DNA labeling, and the hybridization signal can be detected as an anodic current. The average anodic currents of 9 (Cp), 5 (Hb), 8 (Hh) and 7 (MHV) PCR positive samples derived from feces of spontaneously infected mice (Cp, Hb and Hh) and MHV-contaminated tumor cells were 27.9±7.2, 31.9±8.1, 29.3±10.1, and 27.6±3.0 nA, respectively. On the other hand, the average anodic currents of 19 (Cp), 27 (Hb), 18 (Hh), and 13 (MHV) PCR negative samples were 0.3±2.9, 3.7±2.4, −1.0±1.7, and −2.3±2.7 nA, respectively. The anodic current increased with increasing concentrations of pathogens. For experimentally infected samples, the results of PCR/electrophoresis were in complete accord with those of this system when anodic currents of 6.1 (Cp), 8.5 (Hb), 2.4 (Hh), and 3.1 nA (MHV) were taken as the cut-off value. The results suggested that the electrochemical DNA chip system is useful for specific and quantitative detection of PCR products.

Keywords: *Clostridium piliforme*; DNA chip; Electrochemical; *Helicobacter bilis*; *Helicobacter hepaticus*; Mouse; Mouse hepatitis virus; PCR

1. Introduction

Clostridium piliforme (Cp), *Helicobacter bilis* (Hb), *Helicobacter hepaticus* (Hh), and mouse hepatitis virus (MHV) are the most common contaminants in not only laboratory rodents in conventional colonies but also in biomaterials such as tumors and cell lines in Japan (Kagiyama et al., 1987; Goto et al., 2001). The polymerase chain reaction (PCR) has been used as a diagnostic method for these pathogens (Goto and Itoh, 1994; Goto et al., 1995, 2000) because culture of these bacteria from samples is complicated, and MHV isolation by the cell culture method is time-consuming. Although PCR is known to be the most sensitive method for the detection of these pathogens in clinical samples, it has some drawbacks, including false-positive results. To confirm the PCR results, validation methods of the PCR results such as DNA hybridization with specific probes and sequencing of the PCR products are needed.

DNA microarray based experiments are now widely used in many fields. The method of genomic hybridization is applied in the field of single-nucleotide polymorphism (SNP) haplotype analysis (Gentalen and Chee, 1999), identification and classification of genomic alterations in cancer research (Giordano et al., 2005) and detection and genotyping of pathogens (Oh et al., 2004). A combination of multiplex PCR and microarray hybridization is effective for detection of specific pathogens (Fujimuro et al., 2006), and this method can also be used for validation of PCR results. With these methods, probe DNA must be labeled with fluorescent dye such as Cy5 and the fluorescent signal scanned to detect the hybridized products.

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Recently, we developed a microfabricated disposable-type electrochemical DNA chip (Fig. 1). The DNA chip was reacted with PCR products and electrochemically active minor groove binder Hoechst 33258 solution. It was reported that Hoechst 33258, which is known as a minor groove binder that specifically binds to double stranded DNA, is electrochemically active and useful for electrochemical DNA detection (Hashimoto et al., 1994). Then the anodic peak current derived from Hoechst 33258 was measured by cyclic voltammetry. The electrochemical DNA chip does not require DNA labeling, and the hybridization signal can be detected as an anodic current (Hashimoto and Ishomori, 2001) (Patent P2573443, USP5776672, USP5972692, and EP0478319).

In this study we applied this system to detect PCR products from 16S rRNA sequences of Cp, Hb and Hh, and the nucleocapsid protein gene of MHV, and assessed the usefulness of the system.

2. Materials and methods

2.1. Bacterial and viral strains, and growth conditions

The mouse-derived Cp (MSK strain) (Ikegami et al., 1999) was used in this study. The strain was propagated by inoculating intravenously into mice treated with predonisolone. Hb (ATCC 51630) and Hh (ATCC 51448) were cultured on HP agar (Eiken Chemical Co. Ltd., Tokyo, Japan; brain heart infusion blood agar supplemented with 7% horse blood, 10 mg/L vancomycin, 2500 I.U./L polymyxin B, 5 mg/L trimethoprim, and 2 mg/L amphotericin B), and incubated at 37 °C in a mixture consisting of 85% N_2, 10% CO_2 and 5% O_2. MHV-A59 was used in this study, and the virus was grown in DBT cells at 37 °C as described previously (Kunita et al., 1992).

2.2. Clinical and cell/tumor samples

Twenty-eight fecal samples of mice from 6 facilities including 19 negative and 9 positive samples in Cp-PCR, thirty two fecal samples of mice from nine facilities with 27 negative and 5 positive in Hb-PCR, twenty six fecal samples of mice from 11 facilities including 18 negative and 8 positive samples in Hh-PCR, and twenty tumor cells from 10 facilities including 13 MHV-negative and 7 MHV-positive samples were used.

2.3. Detection of organisms from diluted samples

RNA was extracted from a serially diluted homogenate of Cp infected SCID mouse liver. The number of vegetative forms of organisms in 5 μl of the homogenate spread over a 1-cm^2 square area was estimated by a microscopic count of preparations stained with Giemsa's solution (Shoji et al., 1992). The RNA was extracted from a serially diluted Hb and Hh suspension, and numbers of bacteria (colony forming unit; CFU) were obtained by culture on HP agar. RNA was extracted from a serially diluted supernatant of MHV infected DBT cell medium, and DBT cells were used for propagation and titration (plaque forming units; PFU) of the virus.

2.4. Experimental infection

Jcl:ICR and NOD/SCID/γ_c^{null} (NOG) mice aged four weeks were provided by a commercial supplier (CLEA Japan, Inc., Tokyo, Japan). For experimental infection of Cp, five ICR and five NOG mice were gavaged with Cp suspensions including 1.8×10^5 organisms (day 0). At 3 and 7 days postinoculation, fecal samples were collected from the mice. At 8 days postinoculation, cecum, feces, heart and liver samples were

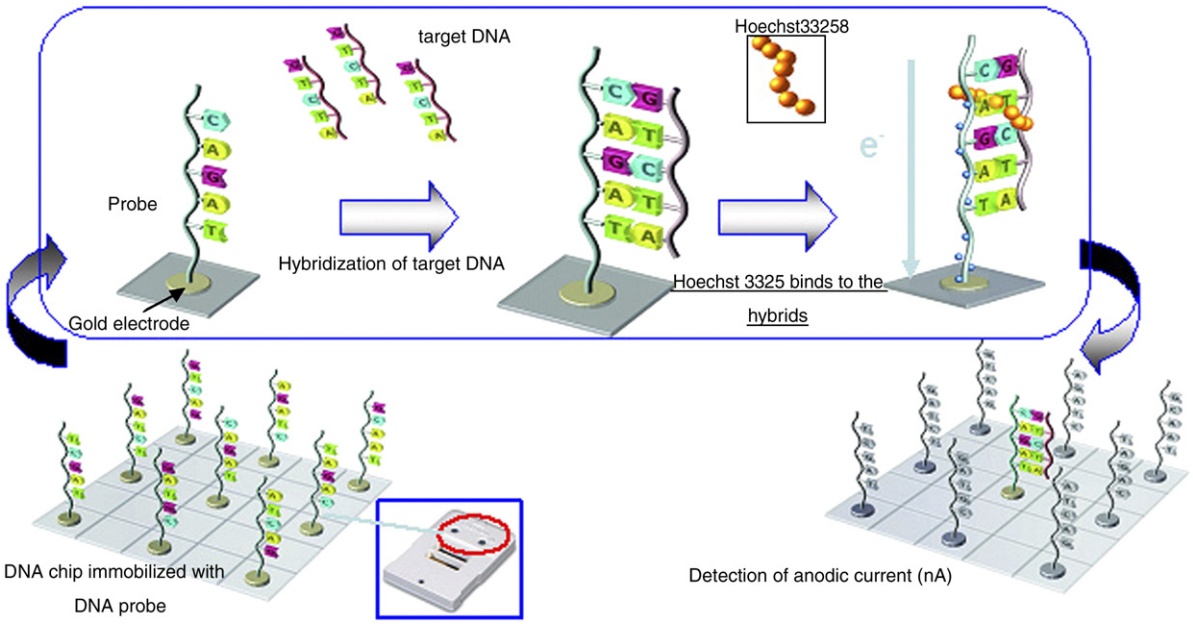

Fig. 1. Principle of the system.