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Original Article

Assessment of *Helicobacter pylori* Viability by Flow Cytometry

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Abstract

Background: Flow cytometry is a rapid, sensitive, and reliable method for determination of bacterial viability. Here we assayed the capability of flow cytometry to detect *Helicobacter pylori* viable cells in both forms of spiral and coccoid. **Methods:** Viable bacteria stained with Rhodamin 123 and fluoresced with laser beam of 488nm. The rate of Rh123 absorption was determined in both forms of bacteria. **Results:** In positive control that consisted of live bacteria, the rate of rh123 absorption was at highest, but negative control that consisted of dead bacteria, the rate of Rh 123 absorption was at lowest absorption. This method showed that non-culturable coccoid forms of *H. pylori*, which could resist environmental stresses, were alive and might be responsible for bacterial transmission and failure in disease treatment. **Conclusion:** Due to simplicity, reliability, and sensitivity of flow cytometry, this method is preferred to other expensive and no reliable methods such as autoradiography, PCR and Electron microscopy used for assessment viability.

Key words: Helicobacter pylori, *Flow Cytometry, Bacterial viability*

Introduction

Helicobacter pylori is a gram negative bacterium which is the causative agent for gastritis, ulcer disease and malignancy (1, 2). The organism exists in two forms, spiral, and coccoid forms (3-5). The coccoid form occurs under unfavorable conditions such a: altered pH, extended incubation, increased oxygen and antibiotic effects (6-8). These forms of bacteria are non-culturalable and it was speculated that coccoid forms were dead cells (9). Recently investigators by using specific methods such as: autoradiography, Electron microscopy and PCR demonstrated that this form of bacteria was alive (6, 10). Because of poor reliability and reproducibility of these methods, they are not wildly used. Therefore, the need for a reliable and rapid method to determine *H. pylori* viability is evident. Flow cytometry allows bacterial viability assessment, in an easy and fast way that measures physical and chemical characteristics of individual cells as they move in a fluid steam past optic and electronic sensors (11).
The aim of this study was verifying the capability of flow cytometry for determination of *H. pylori* viability.

Materials and Methods

Isolation and cultivation The *H. pylori* strains used in this study were isolated from gastric biopsy materials of patients. Bacteria were cultured on Columbia Agar (Difco. Lab) supplemented with 10% horse serum and incubated in microaerophilic conditions (5% O_2, 10% CO_2, 85% NO_2) for 2-3 d. Isolated bacteria were identified according to Linholm et al. (8). Strains

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subcultured in *Brucella* broth (Difco. Lab) supplemented with 6% FCS (Fetal Calf Serum) and incubated in micro-aerophilic conditions. Bacteria that were grown in liquid medium for 2-3 d were 90% in spiral form. To obtain coccoid form of *H. pylori*, incubation of bacteria in liquid medium was continued for more than 10 d. Bacterial morphology was observed by gram staining and microscopy observation before application.

Positive and negative control Positive control consisted of bacterial suspensions in both form (spiral and coccoid) as alive bacteria. Bacteria were killed with 5% sodium hypochlorite made negative control. We also used one strain of *E. coli* obtained from urinary tract and prepared two controls for it: positive control was obtained from fresh culture of *E. coli* in nutrient agar. The same bacteria were killed with 50% sodium hypochlorite and used as a negative control.

Bacterial labeling by Rhodamin 123 All bacterial suspensions were adjusted to 10^7/ml and washed 3 times by PBS (5ml, pH8) and centrifuged 1000g for 10 min. Then bacterial sediments were stained with 2ml Rh123 (Rh 123 Sigma Co.) (EDTA 1ml, pH8) for 30 min.

Flow cytometry and bacterial analysis
FACS caliber (Becton Dickinso) instrument with Helium laser beam (488nm) was used FSC (360 volt), SSC (480volt) and PMT3 (600volt) parameters were selected.

Results

As shown in Fig.1 (a), all analysis were done on the gated bacteria (R), Fig. 1(b) shows the positive control of *H. pylori* spiral forms shows that 92% of bacteria are alive with highest rate of RH123 absorption are at lowest concentration (c). In positive control of *H. pylori* coccoid forms, 94.98% of bacteria had absorbed Rh 123 (Fig. 2a), but the negative control shows that 3.14% of bacteria were alive and had Rh123 absorption (2b). In *E. coli* that used as a bacterial control, results are similar to *H. pylori* spiral and coccoid forms. Positive control of *E. coli* had highest rate of Rh 123 absorption (Fig. 3a) but negative control had lowest concentration of absorbed Rh 123. In addition, we compared the rate of Rh 123 absorption in positive control of forms, spiral, and coccoid (1b, 2a). In spiral form, the rate of absorbed Rh 123 was 2-3 times more than coccoid form.

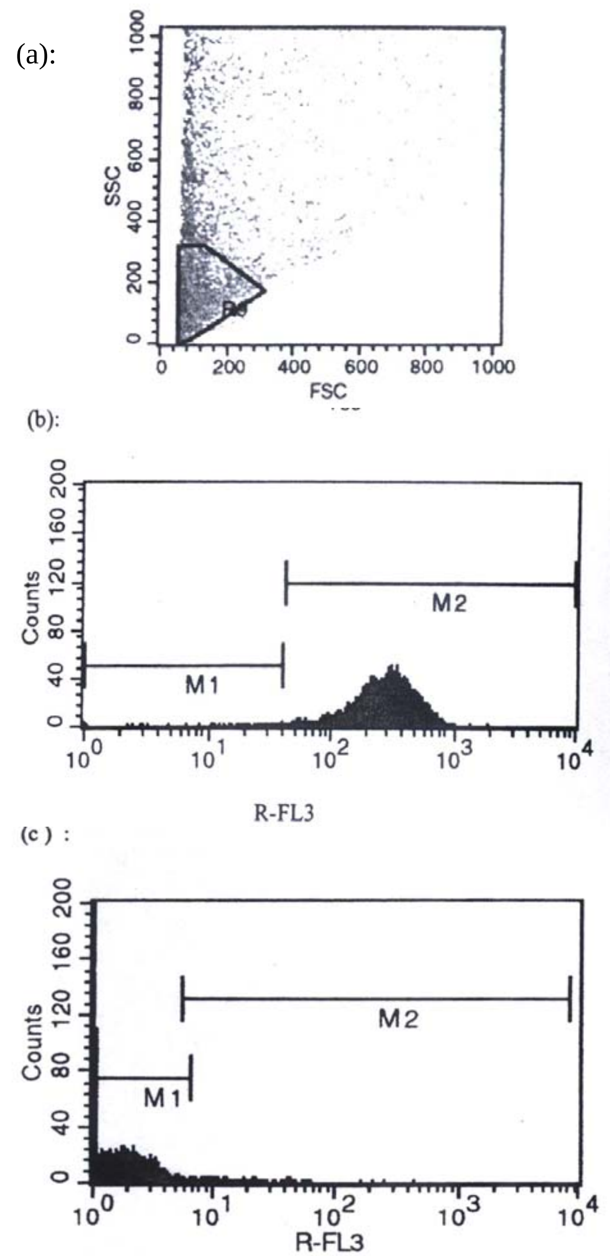


Fig. 1: (a): light scatter of gated cells of *H. pylori*, (b): the rate of Rh 123 absorption in positive control of spiral forms. M1= 2/15%, M2= 92%. (C): The rate of Rh 123 absorption in negative control. M1= 97.18%, M2= 3.55%. SSC: Side scatter of gated cells. FSC: Forward scatter of gated cells: M1: The percent of dead bacteria. M2: The percent of alive bacteria. R-FL3: Light scatter of red fluorescence.

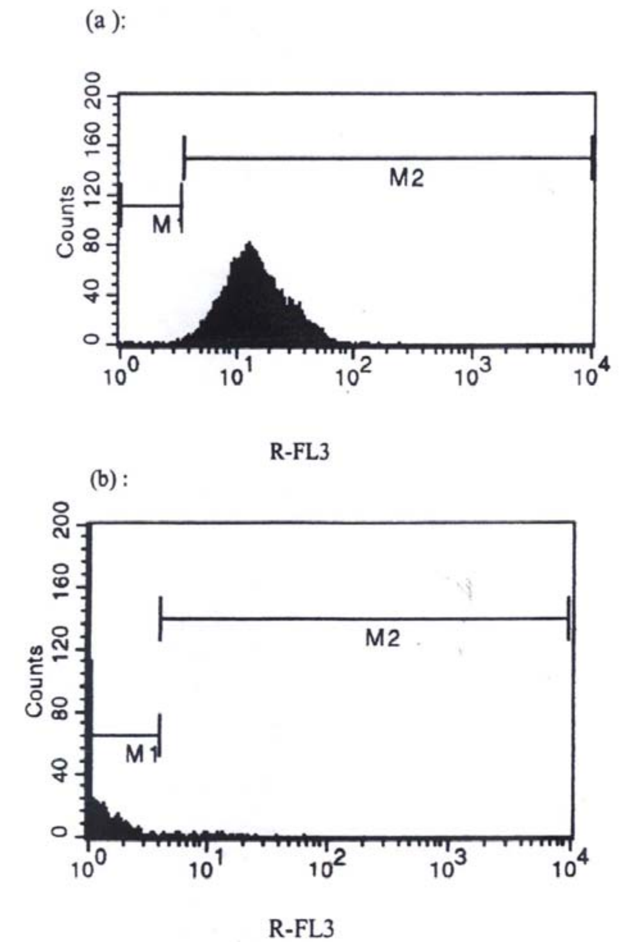
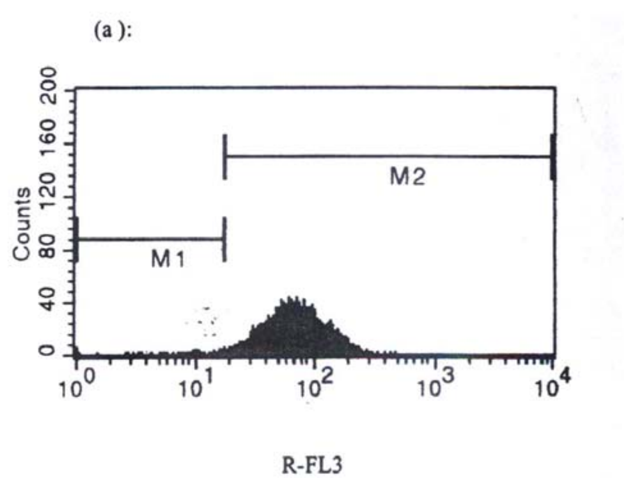


Fig. 2: (a): the rate of Rh123 absorption in positive of coccoid forms. M1= 4.16%, M2=94.98%. (b): The rate of Rh 123 absorption in negative control of coccoids. M1= 96.85%, M2= 3.14%. M1: The percent of dead bacteria, M2: The percent of alive bacteria. R-FL3: Light scatter of red fluorescence.



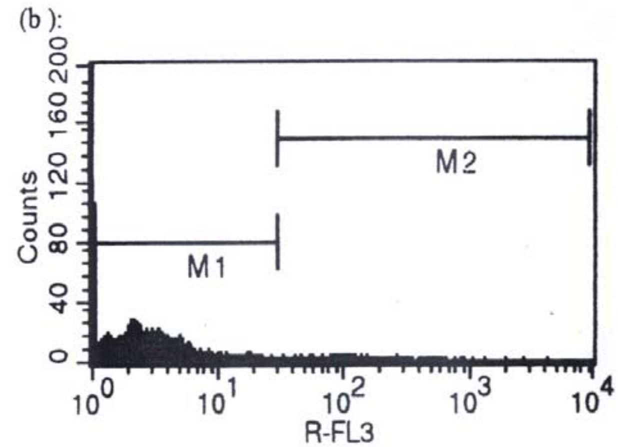


Fig. 3: The rate of absorbed Rh 123 in positive control of E-coli. M1= 2.78%, M2= 98.6%. (b): the rate of Rh 123 absorption in negative control of *E. coli*. M1= 87.17%, M2= 2.04%. R-FL3: Light scatter of red fluorescence. M1: The percent of dead bacteria. M2: The percent of alive bacteria.

Discussion

Resenick has used flow cytometry to determine viability of Mycobacterium smegmatis in 1982 (12). This method is very rapid, sensitive, and reliable (11). Morgan also preferred this method to others because of the same above-mentioned favorites (13). Here, we used flow cytometry for *H. pylori* viability, which had been identified as a causative agent of peptic ulcer and other serious disease (5). This bacterium appears in two forms: spiral and coccoid form (7). The viability of the coccoid form became as an enigma (3). Some authors speculated that it is a morphological manifestation of cell death, but others believe that they are alive cells and have metabolic activity. Many investigators by using difficult and sophisticated methods such as: Electron microscopy, Autoradiography, Probe hybridization and PCR, demonstrated the viability of the coccoid form.

The assessment of *H. pylori* viability by flow cytometry is reported here for the first time. As shown in Fig. 1 (b, c) viable spiral form of *H. pylori* is well differentiated from killed bacteria according to the high Rhd absorption. Rhd is retained in viable bacteria due to their respiration and metabolic activity, whereas deed bacteria are unable to retain Rhd. Fig. 2 (a) showed that coccoids also had absorbed Rhd, which indicated their viability. The rate of Rhd absorption is interesting. Spiral form has Rhd absorption more than coccoid forms, which is attributed to their higher metabolic activity. Our results show that coccoid forms of *H. pylori* are viable cells but they are at lowest metabolic state. This may improve their capability to resist environmental stresses. In Fig. 3 (a), we can see results obtained from *E. coli* as a control bacterium, where viable cells are able to retain Rhd inside bacterium. In this study, we also compared the effect of Azythomycine on spiral and coccoid forms of *H. pylori* viability. By using Azythromycine MIC concentration after 4, 12, and 24 h, the drug was able to kill spiral forms ob bacteria but cocoid forms resisted even after 24 h. Therefore, it is speculated that this form of bacteria is morphological manifestation of resistance and maybe involved in bacterial transmission and of *H. pylori*, changes in morphology, intra cellular composition and surface properties.

In Conclusion because of simplicity, sensitivity, reliability, and high speed of flow cytometry, this is a convenient method for assessment of *H. pylori* viability, especially for the coccoid form. This method will facilitate the assessment of environmental, physical, chemical, and antibiotic effects on *H. pylori* viability.

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