Quantitative Fluorescence In Situ Hybridization of *Bifidobacterium* spp. with Genus-Specific 16S rRNA-Targeted Probes and Its Application in Fecal Samples

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Three 16S rRNA hybridization probes were developed and tested for genus-specific detection of *Bifidobacterium* species in the human fecal flora. Variable regions V2, V4, and V8 of the 16S rRNA contained sequences unique to this genus and proved applicable as target sites for oligonucleotide probes. Determination of the genus specificity of the oligonucleotides was performed by whole-cell hybridization with fluorescein isothiocyanate-labelled probes. To this end, cells were fixed on glass slides, hybridized with the probes, and monitored by videomicroscopy. In combination with image analysis, this allowed quantification of the fluorescence per cell and objective evaluation of hybridization experiments. One of the probes developed was used to determine the population of *Bifidobacterium* spp. in human fecal samples. A comparison was made with results obtained by cultural methods for enumeration. Since both methods gave similar population estimates, it was concluded that all bifidobacteria in feces were culturable. However, since the total culturable counts were only a fraction of the total microscopic counts, the contribution of bifidobacteria to the total intestinal microflora was overestimated by almost 10-fold when cultural methods were used as the sole method for enumeration.

The human intestinal tract harbors an active and complex bacterial ecosystem. The composition and activity of this indigenous gut flora are of paramount importance to human immunology, nutrition, and pathogenesis and hence to the health of the individual (29). Colonization resistance, or bacterial antagonism of the indigenous intestinal microflora, for example, represents a first line of defense against the establishment of pathogenic microorganisms in the intestinal tract (28, 29). The population structure of the intestinal flora influences the effectiveness of colonization resistance greatly (28). Traditional methods to determine the composition of the microflora require cultivation on selective media, which is laborious, time-consuming, and prone to statistical and methodological errors. For this reason, the dynamics of the population composition are difficult to monitor, which hampers objective evaluation of gut flora modulation studies.

In recent years, 16S rRNA probe hybridization has become widely adopted for detection of specific bacterial groups in mixed populations (2, 3, 24, 25, 30, 31). This method is based on the hybridization of synthetic oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation. The specificity of the probe can be adjusted to fit any taxonomic ranking, from kingdom to subspecies (1, 9, 20). DNA hybridization probes have been applied successfully to the intestinal ecosystem. In human feces, *Bacteroides* and *Eubacterium* can be detected by hybridization with a chromosome-targeted probe (12), and in the feces of mice, 16S rRNA-targeted probes have been shown to be applicable for the detection of *Fibrobacter intestinalis* (1). Species-specific 16S rRNA probes have been developed for five *Bifidobacterium* spp. of the human intestinal microflora (35), but the in situ application of these probes has not yet been reported.

In the human intestinal microflora, *Bifidobacterium* is the third most common genus after the genera *Bacteroides* and *Eubacterium* (7). The potential use of bifidobacteria in the treatment and prevention of gastrointestinal disorders (18) has raised the demand for an accurate and easy method for their detection and enumeration. In contrast to the genera *Bacteroides* and *Eubacterium*, the bifidobacteria form a monophyletic cluster on the basis of 16S rRNA sequences (13). This facilitates the development of genus-specific 16S rRNA probes. In studies of the compositional dynamics and metabolic activity of the complete gut flora, genus-specific probes for the identification of distinct physiological groups would combine rapid monitoring with useful taxonomic resolution; i.e., when applied in concert, a limited number of genus-specific probes could be used to describe the overall composition and metabolic potential of the total population. In this communication, we report the development of genus-specific 16S rRNA hybridization probes for the detection of bifidobacteria in human fecal samples.

For the detection of fluorescent oligonucleotide probes hybridized to bacteria on microscopic slides, photography can be applied. However, quantification by this method is hampered by the absence of an objective threshold criterion for discrimination between hybridized and nonhybridized cells. Therefore, for objective evaluation of probe specificity, we have employed an image analysis system which allows fluorometry of individual cells (32–34). In the present study, we have compared 16S rRNA hybridization with classical cultivation techniques for the quantification of bifidobacteria in human fecal samples. The results indicate that virtually all bifidobacteria...
present in feces can form colonies on the Bifidobacterium-selective agar medium employed.

MATERIALS AND METHODS

Probes for in situ hybridization. The 16S rRNA sequences of 18 Bifidobacterium spp. were retrieved from the EMBL and GenBank data libraries (4, 21). Among these were nine Bifidobacterium species which had been isolated from human fecal samples (22). After comparison of unique bifidobacterial sequences with a large number of homologous reference sequences (13), two potential target regions for hybridization probes were selected. The sequences and target sites of the probes are presented in Table 1. Probes Bif164 and Bif662 are complementary to sites in variable regions V2 and V4, respectively (17). A third probe, Bif1278, directed against a site in region V8 was suggested by Brokken et al. (8). The universal-control probe Un519 (9) served as a positive control. The oligonucleotide probes were commercially synthesized and were 5' end labelled with fluorescein isothiocyanate (FITC; Pharmacia Biotech).

Maintenance of cultures. Bacterial cultures were maintained in anoxic chopped meat carbohydrate (CMC) medium (10) at room temperature, with bi-weekly transfers, and in anoxic skimmed milk at −20°C. Prior to hybridization, fresh cultures were grown in anoxic peptone-yeast extract-glucose (PYG) medium (10) at 37°C.

Testing of probe specificity. The bacterial species used for testing the specificity of the probes were derived from human and animal fecal and clinical samples. The probe specificities were examined with 16 species of the genus Bifidobacterium (Table 2) and 20 reference species that are common inhabitants of the human intestinal tract (Table 3). Exponentially growing cells were fixed in ethanol (70%); positive control The oligonucleotide probes were commercially synthesized and were 5' end labelled with fluorescein isothiocyanate (FITC; Pharmacia Biotech).

TABLE 1. Probes used in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Targeta</th>
<th>Sequenceb (5'→3')</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un519</td>
<td>519-536</td>
<td>GTATTACCCCGGCTGTCG</td>
<td>60.2</td>
</tr>
<tr>
<td>Bif164</td>
<td>164-181</td>
<td>CATTGCCGACTCCACCC</td>
<td>60.2</td>
</tr>
<tr>
<td>Bif662</td>
<td>662-679</td>
<td>CACCGTTACCCGGAAGA</td>
<td>60.2</td>
</tr>
<tr>
<td>Bif1278</td>
<td>1278-1294</td>
<td>CCGGTTCGAGGTCATCC</td>
<td>56.6</td>
</tr>
</tbody>
</table>

a Target region for hybridization in the 16S rRNA molecule, numbered according to the homologous E. coli sequence (6).

b Sequence from 5' to 3' end. The 5' end was FITC labelled.

c Tm, theoretical dissociation temperature, based on the formula Tm = 81.5 + 16.6 log[Na+] + 0.41(%G+C) - 820/(probe length), according to reference 13.

d TRI-HCl (pH 7.5), and 0.1% (wt/vol) sodium dodecyl sulfate. After addition of 8 ng of FITC-labelled probe per µl, the smears were covered with a coverslip. The slides were incubated in a buffer-saturated hybridization chamber at 45°C for 15 to 20 h. After hybridization, the slides were washed for 15 min in 50 ml of washing buffer at 80°C. The cell smear was then air-dried, and the cell smears were embedded in mounting fluid, consisting of a 1:1 mixture of glycerol and phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na3HPO4, and 0.24 g of KH2PO4 per liter) supplemented with 2.5% (wt/vol) NaCl.

The slides were evaluated with an Orthoplan epifluorescence microscope equipped with a mercury arc lamp (HBO100W2; Osram, Alphen aan den Rijn, The Netherlands), a 50× PL Fluotar objective (Leitz), an I2/3 (blue excitation) filter block, and a Pelletier cooled charge-coupled device (CCD) video camera (Lori Fullerhead CCD 2500; Lori Fullerhead, Sunnyvale, Calif.). An image analysis software used was the Groningen Reduction of Image Data (GRID) system (14, 34). The fluorescence measurements were performed with the immunofluorescence package described previously (11). Surface fluorescence signals were calibrated by using a nonfading uranyl glass reference (32). Phase-contrast and fluorescence images of each field of view were obtained. The shape of each bacterium was determined from the phase-contrast image automatically. The fluorescence of each object was determined from the corresponding area in the fluorescence image. This procedure avoids exclusion of nonfluorescent objects. The fluorescent images were recorded with a camera exposure time of 12 s. Per microscopic slide, 250 to 500 objects were measured. For each slide, the negative control (autofluorescence) was determined, and the 95th percentile of the fluorescence distribution served as a threshold. The percentage of positively hybridized objects, with fluorescence above the threshold, per total number of objects detected under phase-contrast illumination was doubled the hybridization percentage. This percentage was determined to evaluate the performance of the probes and was done on pure cultures. For graphic representation, surface fluorescence intensity was calculated, and a cumulative Gaussian distribution of the probability density distribution (5) of individual objects, and the maxima of probability density were scaled to 1.00 for presentation purposes.

Enumeration of bifidobacteria and total anaerobes in feces. For quantification of bifidobacteria and total anaerobes in feces, stool specimens from healthy human volunteers were collected and processed as described previously (15). The cultural counts of bifidobacteria were determined on preduced agar-solidified Bacteroides medium (BIF) (27). Since this medium is not highly selective, only those colony types that hybridized positively with probe Bif164 in a separate colony hybridization test were counted. For colony type, these tests were performed in triplicate with cell smears on glass slides as described above. To improve stringency, the hybridization and washing temperature was increased to 50°C. The total number of culturable anaerobes was assessed on prereduced (10% dimethyl sulfoxide) brucella agar plates (18) with anaerobic base (Oxoid, Basingstoke, England) supplemented with 5% (vol/vol) sheep blood, 1 mg of vitamin K1 per ml, and 5 µg of hemin per ml as described before (26).

The slides were evaluated with an Orthoplan epifluorescence microscope equipped with a mercury arc lamp (HBO100W2; Osram, Alphen aan den Rijn, The Netherlands), a 50× PL Fluotar objective (Leitz), an I2/3 (blue excitation) filter block, and a Pelletier cooled charge-coupled device (CCD) video camera (Lori Fullerhead CCD 2500; Lori Fullerhead, Sunnyvale, Calif.). An image analysis software used was the Groningen Reduction of Image Data (GRID) system (14, 34). The fluorescence measurements were performed with the immunofluorescence package described previously (11). Surface fluorescence signals were calibrated by using a nonfading uranyl glass reference (32). Phase-contrast and fluorescence images of each field of view were obtained. The shape of each bacterium was determined from the phase-contrast image automatically. The fluorescence of each object was determined from the corresponding area in the fluorescence image. This procedure avoids exclusion of nonfluorescent objects. The fluorescent images were recorded with a camera exposure time of 12 s. Per microscopic slide, 250 to 500 objects were measured. For each slide, the negative control (autofluorescence) was determined, and the 95th percentile of the fluorescence distribution served as a threshold. The percentage of positively hybridized objects, with fluorescence above the threshold, per total number of objects detected under phase-contrast illumination was doubled the hybridization percentage. This percentage was determined to evaluate the performance of the probes and was done on pure cultures. For graphic representation, surface fluorescence intensity was calculated, and a cumulative Gaussian distribution of the probability density distribution (5) of individual objects, and the maxima of probability density were scaled to 1.00 for presentation purposes.

Table 2. Bifidobacterium species used in this study

<table>
<thead>
<tr>
<th>Bifidobacterium species</th>
<th>Sourcea</th>
<th>Present in human fecesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. adolescentis</td>
<td>NIZO B659</td>
<td>+</td>
</tr>
<tr>
<td>B. angulatum</td>
<td>NIZO B664</td>
<td>+</td>
</tr>
<tr>
<td>B. animalis</td>
<td>LMG 3303</td>
<td>−</td>
</tr>
<tr>
<td>B. asteroides</td>
<td>NIZO B657</td>
<td>−</td>
</tr>
<tr>
<td>B. bifidum</td>
<td>ATCC 29521</td>
<td>+</td>
</tr>
<tr>
<td>B. bovum</td>
<td>NIZO B665</td>
<td>+</td>
</tr>
<tr>
<td>B. breve</td>
<td>LMG 3035</td>
<td>−</td>
</tr>
<tr>
<td>B. cornutum</td>
<td>LMG 3208</td>
<td>−</td>
</tr>
<tr>
<td>B. dentium</td>
<td>ATCC 27678</td>
<td>+</td>
</tr>
<tr>
<td>B. globosum</td>
<td>ATCC 29865</td>
<td>+</td>
</tr>
<tr>
<td>B. infantis</td>
<td>ATCC 15697</td>
<td>+</td>
</tr>
<tr>
<td>B. longum</td>
<td>LMG 3277</td>
<td>+</td>
</tr>
<tr>
<td>B. magnus</td>
<td>NIZO B668</td>
<td>+</td>
</tr>
<tr>
<td>B. pseudolongum</td>
<td>NIZO B669</td>
<td>+</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>NIZO B672</td>
<td>+</td>
</tr>
<tr>
<td>B. suis</td>
<td>NIZO B673</td>
<td>+</td>
</tr>
</tbody>
</table>

a NIZO, Netherlands Institute for Dairy Research, Ede, The Netherlands; LMG, Department of Medical Microbiology, University of Groningen, Groningen, The Netherlands; ATCC, American Type Culture Collection, Rockville, Md.

b According to reference 22.
With the same fecal samples, bifidobacteria were quantified by fluorescence in situ hybridization with the genus-specific probe Bif164 at 50°C. One gram of homogenized feces was suspended in 9 ml of 0.2-μm-pore-size-filtered PBS. This suspension was diluted 10 times in filtered PBS and thoroughly mixed. After removal of debris (35 × g, 15 min), the supernatant was collected and fixed overnight at 4°C with 4% (wt/vol) fresh paraformaldehyde solution. Cells from 1 ml of fixed-cell suspension were washed twice (8,000 × g, 5 min) in 1 ml of hybridization buffer, cells were filtered on a 0.2-μm-pore-size Isopore polycarbonate membrane filter (Millipore Corporation) and washed twice with 10 ml of warm (50°C) hybridization buffer, and 5 μl of FITC-labelled probe was added. Cells were hybridized for 40 h at 50°C. After resuspension in 1 ml of a mixture of PBS and ethanol (1:1), and a UV excitation filter block and by the direct microscopic clump count (DMCC) method (10), During Gram stain preparation for DMCC counts, very mild washings were applied. All microscopic counts were determined in duplicate, with a minimum of 300 cells counted per assay. Statistical analysis included the Student t test and F test.

RESULTS

Selection of target regions. The proposed probe Bif1278 contained one mismatch for B. adolescentis, B. coryneforme, and B. cuniculi. All non-Bifidobacterium species in the RDP database (13) had at least three mismatches. Probe Bif662 was complementary to the 16S rRNA of all Bifidobacterium species. Three Chlorobium species and Gardnerella vaginalis contained one mismatch, and several bacterial species had two mismatches for this probe. None of these bacteria represented known inhabitants of the human intestinal tract. All other sequences in the RDP database contained three or more mismatches for probe Bif662. Probe Bif164 matched with 16 Bifidobacterium spp. in the database. It had one mismatch for B. coryneforme, B. cuniculi, Pseudomonas diminuta, and G. vaginalis, but none of these species are normally encountered in human intestinal flora (7, 22). Furthermore, this probe had two mismatches for Bacillus cycloheptanicus and three mismatches for a number of bacteria, none of which were known representatives of the human intestinal flora. All other organisms in the RDP database had more than three mismatches with probe Bif164.

Probe specificity. After whole-cell hybridization and recording by the CCD camera, fluorescence images, as shown in Fig. 1, were obtained. The fluorescence within the cells was clearly distinct from the dark background and was readily quantifiable by the GRID system. With these quantitative data, a probability density distribution as a function of the fluorescence intensity of individual cells was estimated. Figure 2 is an example of such a plot. In this case, B. adolescentis was hybridized with all three Bifidobacterium-specific probes (Fig. 2a). The fluorescence distribution obtained with probes Bif164 and Bif662 could readily be distinguished from that of the negative control (Fig. 2b). The distribution obtained with probe Bif1278, which contains one mismatch for B. adolescentis, could not be distinguished from that of the negative control. The mean
fluorescence signals (± standard error of the mean [SEM]) for all *Bifidobacterium* spp. hybridized at 45°C with probes Bif164, Bif662, Bif1278, Uni519, and the negative control were 1.381 ± 0.182, 0.866 ± 0.199, 0.215 ± 0.029, 0.919 ± 0.098, and 0.075 ± 0.011, respectively.

The hybridization percentages offer a more objective measure for analysis of probe specificities. Pure cultures of the *Bifidobacterium* spp. that are important members of the human fecal flora, i.e., *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*, exhibited hybridization percentages with Bif164 at 45°C that exceeded 81%. These values were similar to those obtained with Uni519. With probe Bif662, *B. infantis* and *B. breve* exhibited hybridization percentages of 34 and 59%, respectively. The other bifidobacteria from human feces yielded hybridization percentages of greater than 68% with this probe at this stringency. Probe Bif1278 yielded low fluorescence levels, resulting in low hybridization percentages. For bifidobacteria, hybridization percentages with probe Bif164 were significantly higher than with probes Bif662 and Bif1278. For all of the 16 *Bifidobacterium* spp. tested at 45°C, the mean hybridization percentages (± SEM) with probes Bif164, Bif662, Bif1278, and Uni519 were 83% ± 2%, 73% ± 4%, 34% ± 4%, and 83% ± 2%, respectively. Exceptions were *B. cornutum* and *B. globosum*, which showed extremely low fluorescence after hybridization with all probes, including the universal probe. Another species, *B. magnum*, showed an exceptionally low hybridization percentage only with probe Bif164 (37%). Hybridization of the *Bifidobacterium* probes with non-*Bifidobacterium* spp. at 45°C resulted in levels of fluorescence comparable to the negative control values.

The level of fluorescence obtained with Uni519 varied considerably between species (Fig. 3). Although the universal probe resulted in low levels of fluorescence relative to values obtained with *Bifidobacterium*-specific probes, the hybridization percentage of most species tested was more than 80% (Fig. 4). The mean hybridization percentage of nonbifidobacteria with probes Bif164, Bif662, and Bif1278 at 45°C was less than 10%, on average. A typical result, such as that for *Bacteroides* spp., yielded hybridization percentages of 8, 15, and 15% with Bif164, Bif662, and Bif1278, respectively (Fig. 4). The universal probe Uni519 resulted in a value of 91% in this case.

**Quantification of bifidobacteria in feces.** Colony hybridization tests with probe Bif164 showed that BIF agar was indeed not fully selective for bifidobacteria when culturing fecal samples. The number of non-*Bifidobacterium* colonies that developed on BIF agar varied between the samples and was generally between 0 and 10% of the total number of CFU. The mean number (± SE) of culturable bifidobacteria from all samples was 2.45 (± 1.40) × 10⁹ per g of wet feces (Table 4). The mean number of total culturable anaerobes on BBA from all sam-
Bacteroides Eubact. Bifidobact Clostridium Peptostr. Lactobacillus E.co!i Fusobacterium

**FIG. 4.** Percentage of positively hybridized cells in pure cultures of intestinal bacteria after in situ hybridization with the three *Bifidobacterium*-specific probes and the universal probe. The percentages for the various genera are mean values (± SEM) for the species tested.

TABLE 4. Counts of *Bifidobacterium* spp., total bacteria, and total culturable anaerobes in fecal samples from 10 healthy human volunteers as assessed by 16S rRNA probe hybridization, direct microscopy, and cultivation on agar medium.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th><em>Bifidobacteria</em> (10^9/g)</th>
<th>Total anaerobes (10^10/g)</th>
<th>Total bacteria (10^11/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BIF agar</td>
<td>BBA</td>
<td>DAPI</td>
</tr>
<tr>
<td>1</td>
<td>2.27</td>
<td>1.77</td>
<td>7.13</td>
</tr>
<tr>
<td>2</td>
<td>1.58</td>
<td>1.22</td>
<td>6.15</td>
</tr>
<tr>
<td>3</td>
<td>0.55</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>2.73</td>
<td>1.86</td>
<td>2.67</td>
</tr>
<tr>
<td>5</td>
<td>3.10</td>
<td>3.93</td>
<td>3.73</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>0.56</td>
<td>1.30</td>
</tr>
<tr>
<td>7</td>
<td>3.61</td>
<td>2.70</td>
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<tr>
<td>8</td>
<td>3.22</td>
<td>1.61</td>
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<tr>
<td>9</td>
<td>4.69</td>
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<td>4.76</td>
</tr>
<tr>
<td>10</td>
<td>1.05</td>
<td>3.04</td>
<td>2.91</td>
</tr>
</tbody>
</table>

Mean (coefficient of variation) 2.38 (0.57) 2.45 (1.57) 3.87 (0.47) 2.71 (0.26) 1.06 (0.46)

*Explanation of columns: BIF agar, enumeration by filter hybridization with probe Bifl64; BIF agar, total number of culturable bifidobacteria; BBA, total number of culturable anaerobes; DAPI, total microscopic counts with DAPI as the DNA stain; DMCC, direct microscopic clump count. All values are per gram of wet feces.

*ND, not determined.*
respect to both the maximum attainable fluorescence level and the hybridization percentage. The mean hybridization percentage of the 16 *Bifidobacterium* spp. tested with probe B11278 was only 34% (Fig. 4). The poor performance of the probe may result from the presence of an intramolecular binding site in variable region V8 of the 16S rRNA, as described for *Escherichia coli* (6). Such sites may interfere with probe binding. Therefore, a theoretically suitable probe, such as B11278, may not be appropriate for whole-cell in situ hybridization purposes. This should be tested experimentally.

Hybridization with Uni519 revealed heterogeneity of the fluorescence level among the various species and genera. The fluorescence level for certain *Clostridium* spp. and *Fusobacterium* spp. was relatively high, while that of *Eubacterium* spp. and lactobacilli was relatively low (Fig. 5). Such differences may reflect differences in target region availability, cell permeability, or ribosome content of the cells. Low fluorescence levels in positively hybridized cells can significantly overlap high signals of the negative control (autofluorescence). By setting a threshold at the 95th percentile of the fluorescence distribution of the negative control, a discriminator for positively hybridized bacteria was obtained that ensures objective evaluation of hybridization data. However, the use of such a threshold is by no means optimal, since an overlap of the two fluorescence distributions in combination with a high fraction of potentially positive cells results in an underestimate of the hybridization percentage (23). In the case of *Eubacterium* and *Lactobacillales* spp., for example, the low levels of fluorescence after hybridization with Uni519 resulted in significant overlap with autofluorescence. This probably explains the low hybridization percentages found (cf. Fig. 3 and 4). Conversely, the hybridization percentage will be overestimated when the fraction of positive cells is low, which may explain the small but significant hybridization percentages found in nonbifidobacteria after hybridization with *Bifidobacterium*-specific probes. We are currently working on more advanced statistical analysis of these data and on specific evaluation of the probe signal.

In this study, quantitative determination of hybridized bacteria in feces could not be performed on glass slides because of different adherence characteristics of the various bacterial species present in fecal samples (results not shown). Therefore, membrane filters were used for microscopic observation of cells. Since our image analysis system requires phase-contrast light microscopy for segmentation of bacterial objects, evaluation of hybridization with fecal samples was performed visually. According to some investigators, the majority of the fecal flora is culturable with the *Bifidobacterium*-specific probe. We are currently working on more advanced statistical analysis of these data and on specific evaluation of the probe signal.

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