Relationship between the Presence of 44 Megadalton Plasmid and Calcium Dependency or Autoagglutination to Serotype O3 Strains of Yersinia enterocolitica

Seiji KANEKO, and Tsutomu MARUYAMA

Department of Food Hygiene and Nutrition, Tokyo Metropolitan Research Laboratory of Public Health, 3-24-1 Hyakunichō, Shinjuku-ku, Tokyo 160, Japan

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ABSTRACT. A total of 207 strains of Yersinia enterocolitica serotype O3, isolated from human, pig, dog, cat, rat and pork meat during the period between 1972 and 1984 were examined for the 44-megadalton plasmid DNA, calcium dependency and autoagglutination. Strains positive for the plasmid, calcium dependency and autoagglutination were counted and found to be 164(79.2%), 152(73.4%) and 149 (72.0%), respectively. All the plasmid harboring strains were either calcium dependent or positive for the autoagglutination. All the strains which lacked the plasmid were calcium independent and negative for the autoagglutination except four strains (1.9%) that were positive for the autoagglutination. Despite different sources of Y. enterocolitica, all the plasmid positive strains harbored a single plasmid of 44-megadalton. These plasmids had identical restriction endonuclease digestion patterns, and the number of digestion fragments by BamHI, EcoRI and HindIII were 9, 10 and 13, respectively. Based on these results, it is suggested that the presence of the 44-megadalton plasmid in Y. enterocolitica serotype O3 strains is closely related to calcium dependency and autoagglutination and that this plasmid may be inherent to serotype O3.—KEY WORDS: autoagglutination, calcium dependency, plasmid, Yersinia enterocolitica.


Yersinia enterocolitica is widely distributed in the environment and is isolated not only from the intestines of animals, but also from meat, milk, milk products, vegetables and river water [10, 14, 19]. This bacterium is classified into 57 serotypes by its O antigen [17], and some serotypes are known to be pathogenic to man. Epidemiological studies on mass or sporadic diarrhea cases demonstrated that these pathogens belonged to serotypes O3, O5:27, O8 or O9. Organisms of these 4 serotypes have V and W antigens [3, 4], calcium dependency [4], tissue invasiveness [18], autoagglutination [9], serum resistance [12], absorption of Cango Red [13] and latex particle agglutination [8]. These characteristics are closely related to the presence of 40–50 megadalton (Md) plasmid [8, 13, 18].

Most of the previous studies used clinical isolates and did not fully examine the relationship between plasmids and pathogenic markers in known pathogenic strains of Y. enterocolitica. We attempted to elucidate the relationship between pathogenic O3 strains frequently isolated from various sources in Japan and the presence of the 44 Md plasmid. Simultaneously we investigated the correlation between the pathogenic markers such as calcium dependency, or autoagglutination and plasmid harboring. We also examined restriction endonuclease digestion patterns of the plasmid.

MATERIALS AND METHODS

Bacterial strains: A total of 207 Y. enterocolitica serotype O3 strains were used in this experiment and were comprised of the following: 44 strains isolated from human with
mass and sporadic diarrhea, 114 strains from swine cecum contents obtained from abatoirs, 25 strains from canine cecum contents, 3 strains from feline cecum contents, 5 strains from murine cecum contents and 16 strains from retailed pork. All were isolated between 1972 and 1984 in this laboratory.

Calcium dependency: Magnesium oxalate agar medium (MOX) was prepared as previously described by Higuchi and Smith [6]. Each strain was inoculated to 2 plates of MOX and its calcium dependency was determined after incubation for 48 hours, one plate at 37°C and the other at 25°C.

Autoagglutination: Autoagglutination was studied as previously described by Laird and Cavanaugh [9]. Each strain was inoculated to 2 tubes of the medium which was incubated for 24 hours, one tube at 37°C and the other at 25°C to determine autoagglutination.

Isolation of plasmid DNA: Methods by Kado and Liu [13] and by Birnboim and Doly [2] were combined and slightly revised to prepare plasmid DNA. Strains were inoculated into 10 ml of Brain Heart Infusion Broth (Difco) and incubated for 24 hours at 25°C while shaking. The resultant cultures were centrifugated at 8,000 r.p.m. for 10 minutes. The sediment was suspended in one ml of 89 mM Tris-89 mM boric acid and 2 mM EDTA at pH 8.0 (TBE). Two milliliters of lysis solution (3% SDS, 0.5 M Tris, 0.5% NaOH) was added, and the suspension was heated for one hour at 65°C, followed by the addition of 1.5 ml of 3 M sodium acetate at pH 4.8 and standing for one hour at 0°C. Clear lysate was obtained after centrifugation at 15,000 r.p.m. for 15 minutes. Five milliliters of TBE-saturated phenol-chloroform (1:1) was added, mixed, and centrifuged for 15 minutes at 15,000 r.p.m. to obtain 3 ml of supernatant. To the supernatant 6 ml of cold ethanol was added. The mixture was left standing for 2–3 hours at −20°C, and then centrifuged for 15 minutes at 15,000 r.p.m. to obtain the sediment, which was dissolved in 0.5 ml of 50 mM Tris-HCl (pH 8.0) and 0.1 M sodium acetate. The material was further extracted with 1 ml of cold ethanol, and then left standing for one hour at −20°C, after which it was centrifuged again. The nucleic acid materials thus obtained were dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and used for the electrophoresis.

Detection and restriction endonuclease digestion of plasmid DNA: Submarine electrophoresis (70 V, 20 hours) in 0.7% agarose gel and TBE buffer was run to detect plasmid DNA and compare restriction endonuclease patterns [11]. After the electrophoresis, the gel was stained for 30 minutes with 0.5 µg/ml of ethidium bromide and photographed under an ultra violet lamp.

Three restriction endonuclease of BamHI, EcoRI and HindIII were purchased from Nippon Gene Co. (Toyama, Japan). Plasmid DNA isolated from Y. enterocolitica was completely digested under the conditions described in the instruction sheet, and the digests were examined with the agarose gel electrophoresis.

RESULTS

Incidence of plasmid harboring: Of the 207 Y. enterocolitica serotype O3 strains, 164 (79.2%) harbored the single plasmid of approximately 44 Md [15]. The sources of the plasmid-harboring strains were as follows: 31 of 44 strains obtained from human (70.5%), 93 of 114 strains (81.6%) from pig, 21 of 25 strains (84.0%) from dog, one of 3 strains (33.3%) from cat, all of those from rat (100%) and 13 of 16 strains (81.3%) from pork (Table 1). In case of clinical isolates, plasmid of approximately 44 Md was detected in 20 of the 24 (83.3%) freshly isolated strains (<1 year after the isolation) and in 11 of the 20 (55.0%) stock culture (≥1 year after the isolation).

Calcium dependency: Calcium dependen-
Table 1. Incidence of Plasmid Harboring, Calcium Dependency and Autoagglutination of Yersinia enterocolitica Serotype O3 Strains Isolated from Human, Animals or Pork

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of Tested Strains</th>
<th>No. of Plasmid Harboring (%)</th>
<th>No. of Calcium Dependent (%)</th>
<th>No. of Autoagglutination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>44</td>
<td>31 (70.5)</td>
<td>26 (59.1)</td>
<td>30 (68.2)</td>
</tr>
<tr>
<td>Pig</td>
<td>114</td>
<td>93 (81.6)</td>
<td>86 (75.4)</td>
<td>80 (70.2)</td>
</tr>
<tr>
<td>Dog</td>
<td>25</td>
<td>21 (84.0)</td>
<td>21 (84.0)</td>
<td>21 (84.0)</td>
</tr>
<tr>
<td>Cat</td>
<td>3</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>5 (100)</td>
<td>5 (100)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Pork</td>
<td>16</td>
<td>13 (81.3)</td>
<td>13 (81.3)</td>
<td>12 (75.0)</td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>164 (79.2)</td>
<td>152 (73.4)</td>
<td>149 (72.0)</td>
</tr>
</tbody>
</table>

Table 2. Correlation of Harboring Plasmid, and Calcium Dependency or Autoagglutination of Yersinia enterocolitica Serotype O3 Strains Isolated from Human, Animals or Pork

<table>
<thead>
<tr>
<th>Harboring Plasmid</th>
<th>Calcium Dependency</th>
<th>Autoagglutination</th>
<th>No. of Strains</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>132</td>
<td>63.8</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>—</td>
<td>21</td>
<td>10.1</td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>+</td>
<td>11</td>
<td>5.3</td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>0</td>
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<tr>
<td>—</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<td>—</td>
<td>+</td>
<td>—</td>
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<td>0</td>
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<tr>
<td>—</td>
<td>—</td>
<td>+</td>
<td>4</td>
<td>1.9</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>39</td>
<td>18.8</td>
</tr>
</tbody>
</table>

As shown in Table 2, the correlation between the presence of the plasmid and calcium dependency or autoagglutination was classified into 8 types of combination. Of the 164 strains harboring plasmid, 132 strains (63.8%) were both calcium dependent and autoagglutination positive, 21 (10.1%) were calcium dependent but autoagglutination negative and 11 (5.3%) were calcium independent but autoagglutination positive. However, no strain that was calcium dependent and autoagglutination negative was detected.

On the other hand, all the 43 strains which were found not harboring plasmid DNA were calcium independent, among which 4 (1.9%) were autoagglutination positive and 39 (18.8%) were autoagglutination negative.

Restriction endonuclease digestion patterns of plasmid DNA: Plasmid DNA isolated from Y. enterocolitica serotype O3 was di-
Fig. 1. *HindIII* restriction endonuclease digestion patterns of plasmid DNA from strains of *Y. enterocolitica* serotype 03 isolated from human, animals or pork. 1. Molecular weight marker (lambda DNA+EcoRI+HindIII), 2. 84-47 (human), 3. HY-60 (pig), 4. Te 1398 (dog), 5. Te 713 (cat), 6. Te 1502 (rat), 7. Ysk 8 (pork).
gested with restriction endonuclease HindIII and subjected to 0.7% agarose gel electrophoresis. The results are shown in Fig. 1. Despite different sources all the plasmids were digested into 13 fragments and the molecular weight of each fragment was almost the same. Digestion of each plasmid with restriction endonuclease BamHI and EcoRI produced 9 or 10 fragments, having similar molecular weights.

DISCUSSION

Since Zink et al. [18] first reported that tissue invasiveness of Y. enterocolitica is dependent on 41 Md plasmid, many have investigated the relationship between plasmid and pathogenicity using mainly clinical isolates [1, 4, 5, 8, 12, 13, 16].

However, little is known about the extent of virulence associated plasmid distribution in the non clinical environment. A plasmid of 44 Md molecular weight was detected in 79.2% of Y. enterocolitica serotype O3 isolated from various sources. Calcium dependency was found in 73.4% whereas autoagglutination was confirmed in 72.0% of the strains. Except for the small number of strains such as those obtained from cat and rat, there was no significant difference among the strains isolated from different sources as to the plasmid-harboring, calcium dependency and ratio of autoagglutination positive- ness. It is known from the relation among the above three characteristics that the plasmid harboring in any strain is closely related to calcium dependency and autoagglutination and that plasmid harboring strains are generally calcium dependent and autoagglutination positive or both. Since 1.9% of the strains which were not harboring plasmid were autoagglutination positive, it is shown that calcium dependency is more closely related to plasmid harboring.

Since 44 strains (21.3%) obtained from human, in comparison to 163 strains (78.7%) from other sources, have lower plasmid harboring rate, comparison was made between the fresh strains isolated within one year and stock strains isolated over one year ago. There was a significant difference in the rate of plasmid presence between two categories. The plasmid harbored in Y. enterocolitica, therefore, is likely to be lost during storage. Heesmann et al. [5] pointed out that plasmid was detected in 100% of fresh strains from clinical sources but that there were some stock strains without harboring plasmid. Aulisio et al. [1] demonstrated experimentaly that plasmid was not detected and autoagglutination was negative unless the number of plasmid-harboring cells amounted to over 10% of the number of inoculated cells. These results indicate that it is necessary to accurately select plasmid harboring strains if any characteristics related to plasmid and pathogenicity of Y. enterocolitica are to be studied. Thus it is desirable to establish a proper selection method for plasmid harboring cells.

Plasmids isolated from Y. enterocolitica serotype O3 of different sources were digested with restriction endonuclease BamHI, EcoRI and HindIII into 9, 10 and 13, respectively, and the result demonstrated that similar patterns of fragments were obtained. These plasmids, therefore, are regarded as those inherent to Y. enterocolitica serotype O3 and are known to be widely distributed in the environment.

REFERENCES


要 約

各種材料から分離した Yersinia enterocolitica 血清型O3株の保有する 44Md プラスマドとカルシウム依存性および自己凝集性との関連：金子誠二・丸山 務（東京都立衛生研究所生活科学部）—1972～1984年までの間にヒト、プタ、イス、ネコ、ドブネズミおよび豚肉から分離された Y. enterocolitica 血清型O3株、合計207株を用いての約 44Md プラスマドの検出、カルシウム依存性および自己凝集性の有無を調べた。プラスマド保有株、カルシウム依存性株および自己凝集性陽性株はそれぞれ164株(79.2%)、152株(73.4%)および149株(72.0%)であった。プラスマド保有株はカルシウム依存性あるいは自己凝集性陽性であった。プラスマド非保有株のうち4株(1.9%)が自己凝集性陽性を示し、残りはすべてカルシウム非依存性で自己凝集性陰性であった。Y. enterocolitica は由来が異なるにもかかわらず、44Md の単一のプラスマドを保有していた。これらのプラスマドの制限酵素切断パターンは等しく、BamHI, EcoRI および HindIII でのフラグメント数はそれぞれ9, 10および13であった。これらのことより、Y. enterocolitica 血清型O3の保有する約 44Md のプラスマドは、由来が異なってもカルシウム依存性や自己凝集性と密接な関係にあり、このプラスマドが血清型O3に固有であること事が示唆された。