Evaluation of the virulence potential of *Yersinia enterocolitica* isolates from milk by cell invasion-inhibition assay

Ruchi KUSHAL, Sanjeev K. ANAND*

Division of Dairy Microbiology, National Dairy Research Institute, Karnal-132001, India

Received 29 April 2005 – Accepted 10 October 2005

Abstract – The psychrotrophic nature of *Yersinia enterocolitica* is of particular significance in milk and milk products that are normally refrigerated for considerable duration. Different virulence markers have been suggested to differentiate the non-virulent strains from virulent ones. The previous study conducted in our lab, however, failed to show any consistent correlation amongst these markers. As the process of invasiveness of the strain comprises attachment to the intestinal epithelial cell surface, leading to colonization, cell damage, internalization, disturbance of regulatory cell mechanisms and intracellular proliferation, an attempt was made in the present study to evaluate the virulence potential of *Yersinia enterocolitica* isolates from milk by cell invasion-inhibition assay. The present study revealed the presence of *Y. enterocolitica* in about 32.7% of the raw milk samples. Out of a total of about 36 confirmed isolates, only one isolate was found to be virulent based on the cell invasion-inhibition assay.

milk / *Yersinia* / virulence / cell invasion-inhibition assay

Résumé – Évaluation du potentiel de virulence de souches d’*Yersinia enterocolitica* isolées de lait par un test d’invasion-inhibition cellulaire. La nature psychrotrophe d’*Yersinia enterocolitica* a une importance particulière dans le lait et les produits laitiers qui sont normalement conservés longtemps au froid. Différents marqueurs de virulence ont été proposés pour différencier les souches non virulentes des souches virulentes. L’étude antérieure réalisée dans notre laboratoire n’avait cependant pu démontrer aucune bonne corrélation entre ces marqueurs. Comme le processus

* Corresponding author (通讯作者): anand_sanjeevk@yahoo.co.uk
d’invasion de la souche comporte l’adhésion à la surface des cellules épithéliales de l’intestin conduisant à la colonisation, à la lésion de la cellule, à l’internalisation, à la perturbation des mécanismes régulateurs de la cellule et à la prolifération intracellulaire, un essai a été mis en place dans la présente étude pour évaluer le potentiel de virulence d’isolats d’*Yersinia enterocolitica* provenant de lait par un test d’invasion-inhibition cellulaire. Les résultats révèlent la présence d’*Yersinia enterocolitica* dans environ 32,7 % des échantillons de lait cru. Sur un total de 36 isolats confirmés, un seul s’avérait être virulent.

**lait / Yersinia / virulence / test d’invasion-inhibition cellulaire**

**1. INTRODUCTION**

*Yersinia enterocolitica*, la cause de yersiniosis, est largement distribuée dans l’environnement. Il s’agit l’un des rares agents pathogènes humains qui peuvent se multiplier à des températures de conservation et son présence dans les aliments est de grande importance en santé publique. La psychrotrophie du microorganisme est de particulier signification dans le lait et les produits laitiers qui sont normalement conservés à des durées de temps considérables [1, 4, 7, 15, 17, 20]. De plus grande conséquence est l’évaluation de la virulence des souches [5, 23], car c’est les souches virulentes, bien qu’encore peu fréquemment rencontrées, qui peuvent causer des maladies alimentaires. En effet, la plupart des incidents de *Yersinia* pourraient être non-invasifs et peuvent être présents dans des produits alimentaires sans causer de maladies. Ces souches sont parfois également désignées comme des souches environnementales.

Un certain nombre de schémas ont été proposés pour classifier les souches à au moins cinq biotypes [16]. Parmi ces biotypes, certains ont été accordés un statut spécifique. La référence a souvent été faite au biotype 1, qui a récemment été divisé en 1A, représentant les souches d’origine environnementale qui ne sont pas pathogènes, et 1B, qui sont d’origine humaine et sont pathogènes. En raison de l’importance de la virulence des souches, différents virulence marqueurs tels que la morphologie du colonie sur agar tryptique soya agar, la liaison à la violette, la croissance dépendante du calcium et la réaction d’auto-agglutination ont été suggérés pour différencier les souches non-virulentes des souches virulentes. L’étude précédente effectuée dans notre laboratoire n’a pas montré de corrélation constante entre ces marqueurs [8]. Certains autres auteurs ont signalé la virulence de *Yersinia* dans des cellules HeLa [18, 19] et des cellules épithéliales humaines (HEP 2) [10]. De plus, l’utilisation d’enterovirulents *Y. pseudotuberculosis* [3] et de cytoxicité de diverses espèces [21]. En conséquence, le processus d’invasivité de la souche comprend l’adhésion à la surface des cellules épithéliales de l’intestin conduisant à la colonisation, à la lésion de la cellule, à l’internalisation, à la perturbation des mécanismes régulateurs de la cellule et à la prolifération intracellulaire, un essai a été mis en place dans la présente étude pour évaluer le potentiel de virulence d’isolats d’*Yersinia enterocolitica* provenant de lait par un test d’invasion-inhibition cellulaire. Les résultats démontrent la présence d’*Yersinia enterocolitica* dans environ 32,7 % des échantillons de lait cru. Sur un total de 36 isolats confirmés, un seul s’avérait être virulent.

**2. MATERIALS AND METHODS**

**2.1. Isolation of Yersinia enterocolitica from milk samples**

Un total de 110 échantillons a été testé pour la présence de *Yersinia enterocolitica*. Ces échantillons comprenaient 80 laits crus et 30 laits pasteurisés. Pour avoir une couverture plus large, les échantillons ont été prélevés dans différents villages et étaient la plupart du temps appartenant à des exploitations individuelles. L’organisme a été isolé en suivant la procédure standard impliquant une première étape d’enrichissement par addition de 25 mL d’échantillon bien mélangé dans 225 mL de broth peptoné-sorbitol-bile (PSBB) et incubation à 10 °C pendant 10 d, suivie de traitement alcalin (0,5% KOH dans 0,5% saline), et suivi d’incubation sur plaques de cefsulodin-irgasan-novobiocin (CIN) pour 48 h à 25 °C. Les colonies typiques avec un centre rouge foncé (œil de Bœuf) étaient entourées d’une zone claire et colorée.
entire edge were selected and further identified biochemically for the H$_2$S production, urease test, esculin hydrolysis, lipase test, oxidase test, catalase test and Gram staining [13].

2.2. Adherence assay

The adherence of the representative isolates was observed using light and scanning electron microscopy (SEM), while the quantitative estimation was carried out by cell invasion-inhibition assay.

For adherence studies, Colo-320 DM monolayers were prepared on glass coverslips that were placed in six well tissue culture plates in a CO$_2$ incubator. The monolayers were washed twice with phosphate buffered saline (PBS). A 100-µL sample of the selected isolate was cultured in brain-heart-infusion (BHI) broth for 24 h at 25 °C and was added to each coverslip with a monolayer of cell line. The tissue culture plates with coverslips were incubated at 37 °C in 10% CO$_2$: 90% air atmosphere. After one hour of incubation, the monolayers were washed five times with PBS and fixed with methanol. For light microscopy different staining techniques were followed using Gram stain, Giemsa and light green.

For SEM studies, the tissue culture cells were grown on glass chips (1 cm$^2$). After the bacterial adhesion assay, as adopted for light microscopy, the cells were fixed for 2 h at 4 °C with 2.5% gluteraldehyde in 0.1 mol·L$^{-1}$ phosphate buffer (pH 7.4). After two washes with phosphate buffer, the cells were post-fixed for 1 h with 2% osmium tetra oxide (OsO$_4$) in the same buffer. They were then washed three times with phosphate buffer and dehydrated in graded series of ethanol (30, 50, 70, 60, 90 and 100%). The cells were dried by freeze-drying and were coated with gold. The specimens were then examined with a Hitachi S-405 scanning electron microscope.

2.3. Pathogen cell invasion-inhibition assay

Bacterial internalization was determined by estimation of bacteria located within the cells of the Colo-320 DM cell line using a reference strain of *Yersinia enterocolitica* (MTCC-861, obtained from IMTECH, Chandigarh, India) and the milk isolates. To carry out the invasion-inhibition assay, the Colo-320 DM cell lines were grown on six well tissue culture plates in RPMI-1640 medium supplemented with 10% fetal calf serum. Healthy cells, as observed by the inverted microscope, were taken for further studies. The Colo-320 DM monolayers were washed twice with phosphate buffer saline (PBS) before the assay. About 2.0-mL suspensions of 24-h-old BHI growth of *Yersinia* cultures, obtained at 25 °C, were added to wells of tissue culture plates. The plates were incubated for 2 h at 37 °C in 10% CO$_2$: 90 percent air atmosphere. After incubation, the plates were washed five times with sterile PBS and then incubated for 60 min in a medium containing 100 µg of gentamycin·mL$^{-1}$. Since the gentamycin does not diffuse rapidly into the cells, the bacteria that adhered to the brush borders of Colo-320 DM were rapidly killed. Contrary to this, the bacteria located within the cells of the cell line were not killed. The monolayers were then washed with PBS and lysed with sterilized water. Appropriate dilutions were plated on cefsulodin-irgasan-novobiocin (CIN) agar plates and incubated for 48 h at 25 °C to determine the number of viable intracellular yersiniae. Each assay was conducted in duplicate with two successive passages of Colo-320 DM cells [2].

3. RESULTS AND DISCUSSION

3.1. *Yersinia enterocolitica* milk-borne isolates

The results obtained in the present study indicated the presumptive presence of *Y. enterocolitica* in about 73% of the samples of raw milk. On the other hand, none of the pasteurized milk samples were found to contain the organism.

Of the 69 isolates, selected on the basis of colony morphology, only 58 were identified as presumptive *Y. enterocolitica* based on the biochemical identification
scheme as described above. Similar tests have also been used by other authors for presumptive testing of samples [22]. The presumptive identification of the isolates was followed by the primary characterization and confirmation tests as per Bergey’s Manual [6]. On the basis of these tests about 47 isolates were primarily identified as *Y. enterocolitica*, which were oxidase-negative and catalase-positive. On the other hand, on the basis of additional tests [6], such as the negative decarboxylase test for lysine and arginine, inability to deaminate phenylalanine, motility at 25 °C, indole production, a negative response to citrate utilization and the Voges-Proskauer test (at 37 °C), and a negative or delayed positive for the indole test, only 36 isolates were finally identified as confirmed *Yersinia enterocolitica* isolates. On the basis of the final distribution pattern of these confirmed isolates, it was concluded from the present study that about 32.7% of the raw milk samples were positive for *Yersinia enterocolitica*. There have also been several worldwide reports in the past regarding the incidence of the organism in raw milk that has varied from 13.1% in Sweden to 48.1% in Wisconsin. On the other hand, its reported presence in pasteurized milk has mainly been due to either inadequate heat-treatment or post-pasteurization contamination [1, 4, 7, 15, 17, 20]. Our results also confirmed the adequacy of HTST pasteurization in completely eliminating the organism.

### 3.2. Adhesion and invasive studies

Experiments conducted to establish the infective capacity, using in vitro studies with monolayers of Colo-320 DM cell lines, revealed the potential of the virulent isolates to adhere to human cell lines (Fig. 1). It may be seen that the organisms adhered to the periphery of the cell line, as shown by Giemsa stain, under light microscopy. However, neither Gram or light green stains could achieve the desired results. Our results support the previous report on adherence using light microscopy [9].

In the case of studies using scanning electron microscopy, the interaction of the organism and the surface of cultured cell lines could further substantiate the results on infectivity of the virulent isolates (Fig. 2). As the intestinal epithelial cells act as the first barrier to bacteria, the attachment of pathogenic bacteria to the intestinal epithelial cell-surfaces was reported to be significant for colonization, cell damage, internalization and disturbances of regulatory cell mechanisms [3]. The attachment to

---

**Figure 1.** Adhesion of a virulent isolate to Colo-320 DM cells (Giemsa stain, 1000×).
Milk-borne *Yersinia* 175

the intestinal walls and penetration of the mucosa was also suggested to facilitate the survival and proliferation of the organism in the host tissue [11, 12].

### 3.3. Cell invasion–inhibition assay

The infectivity was also shown directly by plating techniques using a cell invasion–inhibition assay. The data presented in Table I reveals the invasion efficiency (IE%) of the virulent isolate from raw milk was 0.5%, as compared with 0.8 for the reference strain (MTCC 861). The present investigation also revealed that at least one raw milk isolate amongst the 36 confirmed isolates of *Yersinia enterocolitica* showed virulence potential by cell invasion-inhibition assay. A similar IE% was previously also used as an index of infectivity for *Listeria monocytogenes* [14].

### 4. CONCLUSION

The present study reveals the presence of *Y. enterocolitica* in about 32.7% of the raw milk samples. Out of a total of about 36 confirmed isolates, only one isolate was found to be virulent based on the cell invasion–inhibition assay. The study thus emphasizes the importance of establishing the infective properties of the *Yersinia enterocolitica* isolates with techniques such as tissue culturing, prior to formulating a further action plan for its control.

**Table I.** Invasion efficiency (IE%) of Colo-320 DM cell monolayers by virulent strains of *Yersinia enterocolitica*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Multiplicity of infection (cfu·mL⁻¹)</th>
<th>Inoculated</th>
<th>After Gentamycin Treatment</th>
<th>IE%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em></td>
<td></td>
<td>4 × 10²</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>(MTCC 861)</td>
<td></td>
<td>5 × 10²</td>
<td>4</td>
<td>0.80</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td></td>
<td>4 × 10²</td>
<td>2</td>
<td>0.50</td>
</tr>
<tr>
<td>(Raw milk isolate)</td>
<td></td>
<td>6 × 10²</td>
<td>2</td>
<td>0.33</td>
</tr>
</tbody>
</table>

**Figure 2.** Scanning micrograph showing the penetration of a virulent isolate into the peripheral region of Colo-320 DM cells (6000×).
Acknowledgements: The financial assistance provided to the first author in the form of a NDRI Jr. Research Fellowship is gratefully acknowledged by the first author.

REFERENCES


