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Note

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

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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

摘要 – 通过细胞浸润－抑制试验评价从牛奶中分离小肠结肠炎耶尔森氏菌的潜在毒性。小肠结肠炎耶尔森氏菌是一种自然低温菌，存在于一些低温冷藏的牛乳和乳制品中。并不是所有的小肠结肠炎耶尔森氏菌菌株都具有毒性，因此有必要标记出不同小肠结肠炎耶尔森氏菌菌株的毒性。本实验室曾经进行过这方面的研究，但并没有取得突破性的成果。由于耶尔森氏菌菌株在细胞浸润过程中会粘附在肠上皮细胞表面，进而导致菌株在其上定植，造成细胞损伤，细胞内摄作用，干扰细胞的调节机制和细胞内的增殖，因此本研究尝试通过细胞浸润－抑制试验来判断由乳中分离出的小肠结肠炎耶尔森氏菌的潜在毒性。研究表明约 32.7% 的原料牛乳样品中存在小肠结肠炎耶尔森氏菌。经过进一步的分离和验证，证明有 36 株小肠结肠炎耶尔森氏菌，根据细胞浸润－抑制试验证明，36 株菌中只有一株菌具有毒性。

耶尔森氏菌 / 毒性

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, the cause of yersiniosis, is widely distributed in the environment. It is one of the few human pathogens that can grow at refrigeration temperatures and its presence in food is of great public health concern. The psychrotrophic nature of the microorganism is of particular significance in milk and milk products that are normally refrigerated for considerable duration [1, 4, 7, 15, 17, 20]. Of greater consequence is the evaluation of the virulence of strains [5, 23], since it is the virulent strains, though encountered infrequently, that may cause food-borne illness. On the other hand, most of the incidental *Yersinia* could be non-invasive and may be present in food products without causing any ill effects. These strains are sometimes also referred to as environmental strains.

A number of schemes have been proposed to categorize the strains into at least five biotypes [16]. Some of these biotypes have now been accorded a species status. Reference has often been made to biotype 1, that has recently been divided into 1A, representing the strains of environmental origin that are non-pathogenic, and 1B, that are of human origin and are pathogenic. In view of the importance of the virulence status of the strains, different virulence markers such as colony morphology on trypticase soya agar, crystal violet binding, congo red uptake, calcium-dependent growth and auto-agglutination reaction have been suggested to differentiate non-virulent strains from virulent ones. The previous study conducted in our lab, however, failed to show any consistent correlation amongst these markers [8]. Some other authors have reported on the virulence of *Yersinia* strains in HeLa cells [18, 19] and human epithelial (HEP 2) cells [10]. Similarly, the use of human intestinal cell lines such as Caco-2 and Colo-320 has also been reported for studying the invasion of enterovirulent strains of *Y. pseudotuberculosis* [3] and cytotoxicity of selected species [21]. 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 *Y. enterocolitica* isolates from milk by cell invasion-inhibition assay.

2. MATERIALS AND METHODS

2.1. Isolation of *Yersinia enterocolitica* from milk samples

A total of 110 samples was screened for the presence of *Yersinia enterocolitica.* These consisted of 80 raw milk and 30 pasteurized milk samples. In order to have a wider coverage, the samples were drawn from various villages and mostly belonged to single animal holdings. The organism was isolated using the standard procedure involving a primary enrichment step by taking 25 mL of well mixed sample in 225 mL of peptone-sorbitol-bile broth (PSBB) and incubating it at 10 °C for 10 d, followed by alkali treatment (0.5% KOH in 0.5% saline), and subsequent streaking on cefsulodin-irgasan-novobiocin (CIN) agar plates. The incubation was carried out for 48 h at 25 °C and the typical colonies with a deep red center (Bull's eye) with a sharp border surrounded by a clear, colorless zone with an

entire edge were selected and further identified biochemically for the H_2S 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 $mol \cdot L^{-1}$ 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 the *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 $\cdot mL^{-1}$. 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

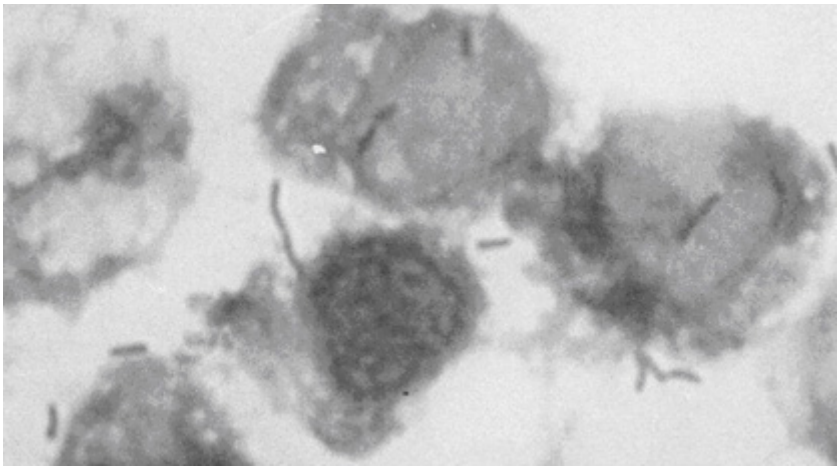

Figure 1. Adhesion of a virulent isolate to Colo-320 DM cells (Giemsa stain, 1000×).

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

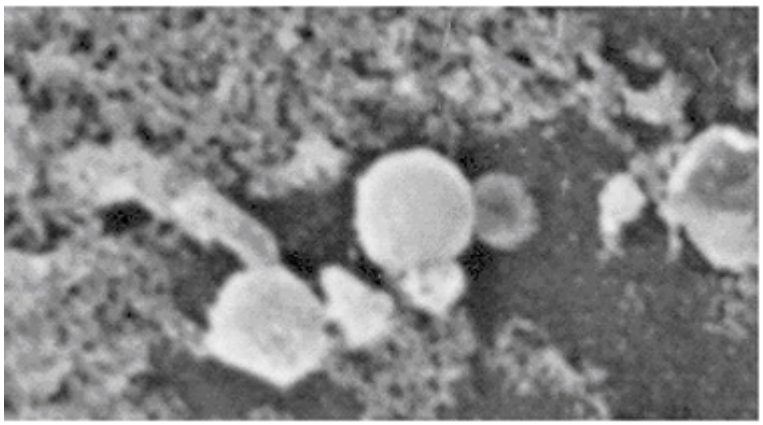

Figure 2. Scanning micrograph showing the penetration of a virulent isolate into the peripheral region of Colo-320 DM cells (6000×).

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

Strains	Multiplicity of infection (cfu·mL^{-1})		
	Inoculated	After Gentamycin Treatment	IE%
Y. enterocolitica	4×10^2	3	0.75
(MTCC 861)	5×10^2	4	0.80
Y. enterocolitica	4×10^2	2	0.50
(Raw milk isolate)	6×10^2	2	0.33

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.

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REFERENCES

[1] Ados R.T., Franco-Bdg-de M., Landgraf M., Incidence of *Yersinia* species in food in Sao Paulo, Brazil, Int. J. Food Microbiol. 21 (1994) 263–270.

[2] Bernet M.F., Brassart D., Neeser J.R., Servin A.L., Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogens-cell interaction, Appl. Environ. Microbiol. 59 (1993) 4121–4128.

[3] Coconnier M.H., Bernet M.F., Kerneis S., Chauvière G., Fourniat J., Servin A.L., Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion, FEMS Microbiol. Lett. 110 (1993) 299–305.

[4] Errico M.M., Pavia M., Prospero E., Grasso G.M., Angelill I.F., *Yersinia* sp. Isolation from raw milk and cheese in Compania, Italy, Ig. Mod. 94 (1990) 212–220.

[5] Greenwood M.H., Hooper W.L., Excretion of *Yersinia* sp. associated with consumption of pasteurized milk, Epidemiol. Infect. 104 (1990) 345–350.

[6] Holt J.G., Krieg N.R., Staley J.T., William S.T., Bergey's Manual of Determinative Microbiology, 9th edn., Williams and Wilkins, Baltimore, USA, 1994.

[7] Huges D., Isolation of *Yersinia enterocolitica* from milk and a dairy farm in Australia, J. Appl. Microbiol. 46 (1979) 125–130.

[8] Kushal R., Anand S.K., A comparison of different virulence markers of *Yersinia enterocolitica*, J. Food Sci. Technol. 38 (2001) 256–528.

[9] Le Chevallier M.W., Schiemann D.A., Macfeters G.A., Factors contributing to the reduced invasiveness of chlorine injured *Yersinia enterocolitica,* Appl. Environ. Microbiol. 53 (1987) 1358–1364.

[10] Maki M., Gronroos P., Wesikari T., Visakorpi J.K., The invasiveness of *Yersinia enterocolitica* in acute diarrhea, Acta Paediatr. Belg. 31 (1978) 108–109.

[11] Mantle M., Husar S.D., Adhesion of *Yersinia enterocolitica* to purified, rabbit and human intestinal mucin, Infect. Immun. 61 (1993) 2340–2346.

[12] Mantle M., Husar S.D., Binding of *Yersinia enterocolitica* to purified, native small intestinal mucins from rabbits and human involves interaction with mucin carbohydrate moiety, Infect. Immun. 62 (1994) 1219–1227.

[13] Marshall R.T., Standard Methods for the Examination of Dairy Products, American Public Health Association, Washington DC, USA, 16th edn., 1993, pp.159–164.

[14] Meyer D.H., Bunduki M., Beliveau C.M., Donnelly C.W., Diffrences in invasion and adherence of *L. monocytogenes* with mammalian gut cells, Food Microbiol. 9 (1992) 115–126.

[15] Moustafa M.K., Ahmed A.A.H., Marth E.H., Behavior of virulent *Yersinia enterocolitica* during manufacture and storage of Colby-type cheese, J. Food Prot. 46 (1983) 318–320.

[16] Nilehn B., Studies on *Y. enterocolitica* with special reference to bacterial diagnosis and occurrence in human acute enteric disease, Acta Pathol. Microbiol. Scand. (Suppl.) 206 (1969) 1–48.

[17] Norberg P., *Yersinia* in pasteurized milk, Var-Foeda 33 (1981) 454–451.

[18] Pederson K.B., Winblad S., Studies on *Yersinia enterocolitica* isolated from swine and dogs, Acta Pathol. Microbiol. Scand. B 87 (1979) 137–140.

[19] Sai T., Nagata K., Kokaa H., A study on the pathogenicity of *Yersinia enterocolitica,* Jap. J. Bacteriol. 30 (1975) 116.

[20] Schiemann D.A., Association of *Yersinia enterocolitica* with the manufacture of cheese and occurrence in pasteurized milk, Appl. Environ. Microbiol. 36 (1978) 274–277.

[21] Smit H.F., Woerdenbag H.J., Singh R.H., Meulenbeld G.J., Labedie R.P., Zwaving J.H., Ayurvedic herbal drugs with possible cytotoxic activity, J. Ethnopharmacol. 47 (1995) 75–84.

[22] Tibana A., Warnken M.B., Nunes M.P., Ricciardi I.D., Noleto A.L.S., Occurrence of *Yersinia* species in raw and pasteurized milk in Rio de Janeiro, Brazil, J. Food Prot. 50 (1987) 580–583.

[23] Walker S.J., Gilmour A.J., A comparison of media and methods for the recovery of *Yersinia enterocolitica* and *Yersinia enterocolitica* like bacteria from milk containing simulated raw milk microflora, Appl. Bacteriol. 60 (1986) 175–183.