

Serological and biochemical characteristics of virulence plasmid of *Yersinia enterocolitica* isolates from chicken in the Islamic Republic of Iran

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الخصائص المصلية والكيميائية الحيوية لبلازميد الفوعة في اليرسينية الملهبة للمعى والقولون المستفردة من الدجاج في جمهورية إيران الإسلامية
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خلاصة: تحتوي اليرسينية الملهبة للمعى والقولون على بلازميد أساسي للفوعة. وقد درسنا خصائص بلازميد الفوعة باختبارات مصلية وكيميائية حيوية ومقاييس حيوية في اليرسينية الملهبة للمعى والقولون المستفردة من الدجاج باستعمال طرائق معالجة البلازميد. وتم الحصول على مشتقات إسوية المنشأ محفوظة البلازميد (C2029 و C2150) من مستفردتين من اليرسينية الملهبة للمعى والقولون (RTCC 2029 و RTCC 2150). وأظهرت النتائج أن المستفردات التي تحمل البلازميد (2029 و 2150) كانت صامدة للمصل البشري عندما زرعت في درجة حرارة 37°C، ولكنها كانت حساسة له عند زراعتها في درجة 25°C. بينما كانت المستفردات ذات البلازميد المستفردة (C2029 و C2150) حساسة للمصل عندما زرعت في كلتا درجتي الحرارة. كما أن اختبارات القراص الذاتي والاعتماد على الكلسيوم، والعدوى التجريبية في الفئران أثبتت أن هذه الأنماط الظاهرية كانت مرتبطة ببلازميد الفوعة.

ABSTRACT Pathogenic *Yersinia enterocolitica* harbour plasmid that is essential for virulence. We studied the characteristics of virulence plasmid using serological, biochemical and bioassay tests in *Y. enterocolitica* isolates of chicken using plasmid curing. Plasmid-cured isogenic derivatives (2029c and 2150c) were obtained from two isolates of *Y. enterocolitica* (RTCC 2029 and RTCC 2150). The results demonstrated that plasmid-bearing isolates (2029 and 2150) were human-serum-resistant when grown at 37 °C, but were sensitive when grown at 25 °C, whereas plasmid-cured isolates (2029c and 2150c) were sensitive when grown at both temperatures. Also autoagglutination, calcium-dependency tests and experimental infection in mice demonstrated that these phenotypes were associated with the virulence plasmid.

Caractéristiques biochimiques et sérologiques du plasmide de virulence des isolats de *Yersinia enterocolitica* réalisés sur des poulets en République islamique d'Iran

RESUME Les *Yersinia enterocolitica* pathogéniques hébergent un plasmide qui est essentiel pour la virulence. Nous avons étudié les caractéristiques de ce plasmide de virulence au moyen d'épreuves de dosage biologique, d'épreuves biochimiques et des sérodiagnostics sur des isolats de *Y. enterocolitica* réalisés chez des poulets utilisant une cure plasmidique. Les dérivés isogéniques ayant subi une cure plasmidique (2029c et 2150c) ont été obtenus à partir de deux isolats de *Y. enterocolitica* (RTCC 2029 et RTCC 2150). Les résultats ont montré que les isolats porteurs de plasmides (2029 et 2150) étaient résistants au sérum humain lorsqu'ils sont cultivés à 37 °C, mais qu'ils étaient sensibles lorsqu'ils sont cultivés à 25 °C, tandis que les isolats traités par plasmides (2029c et 2150c) étaient sensibles aux deux températures. De plus, les épreuves d'autoagglutination, de dépendance au calcium et l'infection expérimentale chez les souris ont montré que ces phénotypes étaient associés avec le plasmide de virulence.

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Introduction

Yersinia enterocolitica has been isolated worldwide from products such as beef, chicken, pork, ice-cream, mussels, oysters and drinking water [1,2]. As a foodborne pathogen *Y. enterocolitica* is a worldwide public health hazard. The dangerous nature of *Y. enterocolitica* is magnified by its ability to survive and multiply in refrigerated foods at 0–4 °C [3]. The clinical manifestations of *Y. enterocolitica* in humans are mostly enteric [4,5]. The predominant clinical features are abdominal pain and diarrhoea that can vary in its severity from a few loose stools per day to a fulminate enterocolitis with ulcerative lesions involving the gastrointestinal tract. Nausea and vomiting can occur but these symptoms are usually not prominent. Abdominal pain is quite common and sometimes evokes an infected appendix. In the latter cases, the symptoms are the result of acute terminal ileitis and severe inflammation of the mesenteric lymph nodes [6,7].

Pathogenic bacteria of *Y. enterocolitica* cause human and animal diseases by invading the host tissues. They harbour a plasmid, which is essential for virulence at 37 °C [8,9]. This virulence plasmid is associated with several virulence characteristics of *Y. enterocolitica*, such as calcium-dependent growth at 37 °C [5], autoagglutination [10] and the production of specific proteins. Plasmid-curing experiments are particularly suitable for the analysis of virulence expression and to identify plasmid-encoded products [11]. The aim of this work was to study the correlation of some biochemical and serological characteristics with virulence plasmid of *Y. enterocolitica* isolated from chicken in the Islamic Republic of Iran using plasmid curing.

Materials and methods

Bacterial strains and growth conditions

Y. enterocolitica isolates RTCC2029 and RTCC2150 (biotype 3) were obtained from the Razi Research Institute for Vaccine and Serum (Karaj, Hesarak, Islamic Republic of Iran). They had been isolated from chicken from various sources, and they harboured virulence plasmid. Their plasmid-cured derivatives, isolates RTCC2029c and RTCC2150c, were obtained as described later in this paper (plasmid-curing section). The bacteria were grown in brain–heart infusion broth (BHI) and BHI medium supplemented with 20 mM magnesium chloride, 20 mM sodium oxalate, and 0.2% glucose (BHI-MOX) at 25 °C and 37 °C.

Plasmid extraction

Plasmid preparation was performed by modification of the Motallebi method [12]. Bacteria from the overnight culture were harvested by spinning for 2–3 minutes in a microcentrifuge (12 000 g). The pellet was resuspended in 100 µL of ice-cold lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). Alkaline sodium dodecyl sulfate (SDS) solution (200 µL, freshly prepared by mixing equal volumes of 0.4 M NaOH and 2% SDS) was added and the mixture vortexed and left on ice for 5 minutes. The majority of the proteins and chromosomal DNA were then precipitated by addition of 150 µL 3M sodium acetate pH 4.8 and mixed by inversion to neutralize the preparation and left on ice for a minimum of 10 minutes. The chromosomal DNA and cell debris were pelleted (5 minute centrifugation at 12 000 g). The supernatant (400 µL) was then removed to a separate tube, mixed with 400 µL of isopropanol and left at 20 °C for 20 min-

utes. The precipitated nucleic acids were then pelleted (12 000 g for 3 minutes), re-suspended in 200 μ L 1 \times TNE (1 mM Tris-HCl, 0.5 M NaCl, 0.05 EDTA), and re-precipitated with 240 μ L isopropanol at -20 $^{\circ}$ C for 20 minutes. After pelleting, precipitation was repeated, then the nucleic acids were spun down, vacuum-dried and resuspended in 50 μ L distilled water.

Chromosomal DNA extraction

Chromosomal DNA was obtained by the method of Eickbush and Moudrianakis [13].

Plasmid curing

Plasmid curing was carried out as described by Shakibaie et al. [11] with some modifications. Trypticase yeast extract (TYE) medium containing curing agents such as ethidium bromide and novobiocine was inoculated with *Y. enterocolitica* strains. Bacteria of the high-passage isolates at elevated temperature (42 $^{\circ}$ C) were serially cultured several times in this medium. Some large colonies grown on BHI medium at 37 $^{\circ}$ C were used for plasmid detection.

Biochemical tests

Salicin and esculin are both β -glucosides that vary in the chemical structure of the compound attached to D-glucose by a beta linkage. Both compounds are hydrolysed by the enzyme β -glucosidase. Fermentation of salicin was determined with 1% salicin and indicator (phenol red broth 1.6%). Esculin hydrolysis was determined in esculin broth (5 g peptone, 1 g KH_2PO_4 , 5 g esculin, 0.5 g ferric citrate as indicator; the total volume was brought to 1000 mL with distilled water). Strains were inoculated, incubated at 25 $^{\circ}$ C and read after 24 hours.

The pyrazinamidase test was conducted as described by Farmer et al. [14]. Bacteria were inoculated over the entire slant of

pyrazinamidase agar, incubated at 25 $^{\circ}$ C for 48 hours, and tested with 1 mL of freshly prepared 1% solution of ferrous ammonium sulfate. A positive pyrazinamidase reaction was indicated by a pink-to-brown colour which developed on the slant, compared with the yellow colour of the control.

Serological tests

Serum resistance was assayed by a modification of the method of Balligand et al. [15]. Approximately 10^7 bacteria of an overnight culture grown at 25 $^{\circ}$ C in BHI were inoculated in 10 mL of BHI contained in a 100 mL conical flask, and grown at 25 $^{\circ}$ C or 37 $^{\circ}$ C with shaking at 180 rpm to a density of 1.5×10^8 cells/mL. The cells were incubated at 37 $^{\circ}$ C in the presence of 5% (v/v) human serum. Viable counts were made by plating appropriate dilutions onto TSA after 0 minutes and 90 minutes.

The ability of *Y. enterocolitica* isolates to autoagglutinate at 37 $^{\circ}$ C was tested by a modification of the method of Prpic et al. [16]. Two 20 mL universal bottles containing 10 mL of TYE were each inoculated with 100 mL of an overnight culture grown at 25 $^{\circ}$ C and incubated at 37 $^{\circ}$ C respectively. The turbidity of the culture was examined after 1–5 hours.

The calcium-dependency test was applied by a modification of the method of Allaoui et al. [17]. The calcium requirement for growth at 37 $^{\circ}$ C was monitored by plating the bacteria in parallel on BHI and BHI-MOX agar.

Results

Plasmid curing

The study of virulence-plasmid-associated functions of *Y. enterocolitica* requires an isogenic pair of strains, one with and one without the virulence plasmid. For plas-

mid-curing experiments, bacteria of the high-passage isolates at 42 °C were serially cultured several times in TYE medium containing novoblocine and ethidium bromide. Strains 2029c and 2150c were obtained from large colonies grown on BHI medium at 37 °C. Chromosomal and plasmid DNA from isolates was loaded on 0.7% agarose gel, separately. The results indicated that the two isolates (2029 and 2150) contained a plasmid which was absent in their isogenic plasmid-cured isolates (2029c and 2150c) (Figure 1). These two pairs of isolates allowed for several plasmid characteristics to be assigned.

Characterization of plasmid-cured isolates

Serological tests

The ability of *Y. enterocolitica* strains to autoagglutinate and to resist the bactericidal activity of human serum at 37 °C and also the calcium requirement for growth at 37 °C (calcium dependency) were tested before and after plasmid curing.

A sample of each of the overnight cultures of *Y. enterocolitica* strains was used for inoculation of BHI medium at 25 °C and 37 °C. The bacteria autoagglutinated after an incubation period of 1 hour at 37 °C. This was seen by the naked eye as a formation of small aggregates in bacterial suspension. Strains 2029c and 2150c did not autoagglutinate at 37 °C whereas strains 2029 and 2150 did (Table 1).

The results of serum resistance tests of strains 2029, 2150, 2029c and 2150c showed that serum resistance was not only virulence-plasmid dependent but also temperature dependent. As expected, strains 2029c and 2150c grown at 25 °C or 37 °C and strains 2029 and 2150 grown at 25 °C were unable to survive 90 minutes after the exposure to 5% human serum at 37 °C (Table 1). On the other hand, strains 2029 and

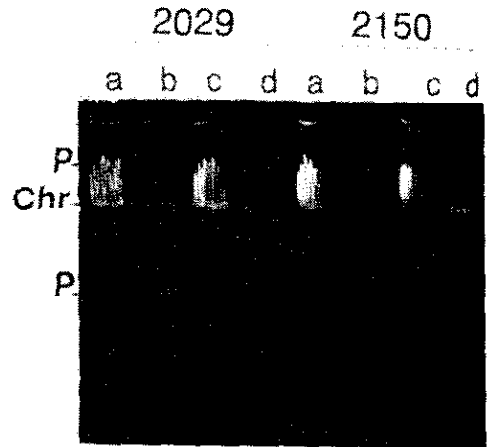


Figure 1 Plasmid and chromosomal DNA extraction in *Yersinia enterocolitica* isolates. Lanes a and c: chromosomal DNA extraction from plasmid-bearing and plasmid-cured isolates respectively. Lanes b and d: plasmid DNA extraction from plasmid-bearing and plasmid-cured isolates respectively. P = plasmid DNA, Chr = chromosomal DNA

2150 grown at 37 °C were able to survive after 90 minutes of exposure to the serum (Table 1).

The results of the calcium-dependency tests of isolates 2029, 2150 and 2029c, 2150c at 25 °C and 37 °C showed that only isolates 2029 and 2150 grew at 25 °C but were unable to grow at 37 °C, whereas isolates 2029c and 2150c grew at both temperatures (Table 1).

Biochemical tests

Salicin fermentation, esculin hydrolysis and pyrazinamidase activity tests were applied to isolates 2029 and 2150 and their isogenic plasmid-cured isolates (2029c and 2150c). The results demonstrated that plasmid-bearing and plasmid-cured isolates had the same behaviour in these tests, indi-

Table 1 Serological tests

Isolate no.	Auto-agglutination		Serum resistance		Calcium dependency	
	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
2029	-	+	-	+	+	-
2150	-	+	-	+	+	-
2019c	-	-	-	-	+	+
2150c	-	-	-	-	+	+

cating that these properties are not associated with the virulence plasmid.

The results of experimental infections in mice showed that infection with plasmid-bearing isolates (2029 and 2150) caused death within 2 days, whereas infection with plasmid-cured isolates (2029c and 2150c) caused neither death nor lesions for 14 days after treatment.

Discussion

Among the 10 isolates of *Y. enterocolitica* isolated from Iranian chicken, 2 isolates were shown to harbour plasmid and to have different serological behaviour than the others (unpublished data). Many different tests and methods have been used over the years to define the pathogenic potential of *Y. enterocolitica* strains, but it is recognized that an important component of virulence in this species is determined by a plasmid [18,19].

The study of virulence-plasmid-associated functions of *Y. enterocolitica* requires an isogenic pair of strains, one with and one without the virulence plasmid. In this work, such pairs were used and the association of the plasmid with serological, biochemical and pathogenicity phenotypes was tested.

The results of the biochemical tests showed that plasmid-bearing isolates (2029

and 2150) were salicin, esculin and pyrazinamidase negative (Sal⁻/Esc⁻/Pyr⁻). These findings are in agreement with the reports of Kandolo and Wauters [18] and Farmer et al. [14] who defined Sal⁻/Esc⁻/Pyr⁻ isolates as potential pathogens. However, in our study, comparison of the results obtained from biochemical tests in plasmid-bearing isolates (2029 and 2150) with their plasmid-cured derivatives (2029c and 2150c) demonstrated that there was no correlation between the biochemical characteristics and the presence of the virulence plasmid.

Furthermore, some reports indicate that calcium-dependent growth at 37 °C [20], serological tests such as autoagglutination, resistance to the bactericidal effect of human serum [9], and the causing of lesions in mice are associated with a virulence plasmid in *Y. enterocolitica* [7]. The results obtained in our study demonstrated that calcium dependency was seen in isolates 2029 and 2150 but not in their cured derivatives (2029c and 2150c). We also showed that plasmid-bearing isolates (2029 and 2150) were resistant to the bactericidal effect of human serum when grown at 37 °C but sensitive when grown at 25 °C, whereas plasmid-cured isolates (2029c and 2150c) were sensitive when grown at both temperatures. Also, autoagglutination was observed in isolates 2029 and 2150 at 37 °C

but not in isolates 2029c and 2150c under the same conditions.

These results concur with the results of Pai and De Stephano [21] and Skurnik et al. [9]. The results of the experimental infections in mice in our study support those of Skurnik et al. [9], who studied experimental infection in guinea pigs, i.e. isolates 2029 and 2150 caused death within 2 days whereas isolates 2029c and 2150c caused neither death nor lesions. Overall, such

findings point to the association of these characteristics with the virulence plasmid of these two isolates (2029 and 2150) from chicken in the Islamic Republic of Iran.

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