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Confirmation of presumptive *Salmonella* colonies contaminated with *Proteus* swarming using the Polymerase Chain Reaction (PCR) method

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ABSTRACT. In México, zero tolerance regulation is practiced regarding *Salmonella* in food products, the presence of which is verified by the procedure described in NOM 114-SSA-1994. During the period between August 2002 and March 2003, 245 food samples were tested using this procedure in the Central Laboratories of the Department of Health for the State of Jalisco (CEESLAB). Of these 245 samples, 35 showed presumptive colonies contaminated with *Proteus* swarm cells even after selective isolation. These swarm cells make *Salmonella* recovery and biochemical identification difficult due to the occurance of atypical biochemical profiles which generally correspond to that of *Proteus*. Out of the 35 samples contaminated with *Proteus*, 65 presumptive colonies were isolated. These colonies were analyzed using both normative microbiological method and Polymerase Chain Reaction (PCR). The PCR method detected two positive samples while normative microbiological method was not able to identify. In order to determine the extent of interference of *Proteus* swarming on the *Salmonella*-specific PCR band amplification, *Salmonella* ser. Typhimurium was grown in the presence of *Proteus* swarming. These results show that *Proteus* swarming did not interfere with *Salmonella* PCR-amplification, although the appearance of *Salmonella* was altered such that the black precipitate was no observed in the presence of *Proteus* swarming. Ours result indicate that the PCR method used in this study may be successfully applied to confirm presumptive *Salmonella* colonies contaminated with *Proteus* swarming.

Key words: Foods, PCR, *Proteus*, *Salmonella*, swarm cells.

RESUMEN. En México las regulaciones sanitarias exigen tolerancia cero para *Salmonella* en productos alimenticios y la presencia de *Salmonella* es verificada de acuerdo con el procedimiento descrito en la NOM 114-SSA-1994. Durante el periodo comprendido entre agosto del 2002 y marzo del 2003, fueron obtenidas 245 muestras de alimento y analizadas utilizando este procedimiento en el Centro Estatal de Laboratorios (CEESLAB) de la Secretaría de Salud. De las 245 muestras, 35 presentaron colonias sospechosas de *Salmonella* contaminadas con swarming de *Proteus* en la etapa de aislamiento selectivo. Este fenómeno dificulta tanto la recuperación como la identificación bioquímica de *Salmonella*, produciendo un perfil bioquímico atípico que generalmente corresponde al de *Proteus*. De las 35 muestras contaminadas con swarming, se recuperaron 65 colonias sospechosas. Estas colonias fueron analizadas por el procedimiento microbiológico y por Reacción en Cadena de la Polimerasa (PCR). La PCR detectó dos muestras positivas que el método microbiológico no fue capaz de detectar. Con la finalidad de determinar el grado de interferencia del swarming en la amplificación de la banda específica de *Salmonella* por PCR, cultivos de *Salmonella* ser. Typhimurium, fueron contaminados de forma artificial con swarming de *Proteus*. Los resultados demostraron que el swarming no interfiere en la reacción de amplificación por PCR, aunque la apariencia de las colonias sospechosas estaba alterada debido a que el precipitado negro característico no se observó en presencia del swarming de *Proteus*, a pesar de estar creciendo en medios selectivos. Estos resultados indican que el método de PCR utilizado en este estudio, puede ser utilizado con éxito para la confirmación de colonias sospechosas de *Salmonella* contaminadas con swarming de *Proteus*.

Palabras clave: Alimentos, PCR, *Salmonella*, swarming de *Proteus*.

INTRODUCTION

Salmonella is one of the most common foodborne pathogens.[2] In México, zero tolerance is required for *Salmonella* in foods products, where detection is achieved through the microbiological procedure is described in NOM-114-SSA1.[11] This method, however, is limited such that it requires 5 to 7 days to obtain positive results,[6] and often the selective isolation media allows other enteric bacteria such as *Proteus* to grow. *Proteus* is an enteric Gram negative rod and some species such a *P. mirabilis* and *P. vulgaris* have active motility at 37° C, which leads to translucent growth over the surface of solid media.[5] This phenomenon, known as "swarming", makes the isolation of presumptive *Salmonella* colonies on solid selective media difficult.[8]

The Polymerase Chain Reaction (PCR) method presents an alternative to the microbial detection described in NOM-114-SSA1. The PCR method allows amplification of specific DNA sequences, which can be used in the

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detection and identification of microorganisms. This method provides high specificity and requires only a day to obtain the result. PCR has been adapted to detect pathogens in food, environmental and clinical samples.[1,3,4,12]

The main purpose of the current study was to evaluate the use of PCR in the confirmation of presumptive *Salmonella* colonies in food samples. The specific objectives were: (i) test the specificity of PCR primers with both *Salmonella* and non-*Salmonella* strains, (ii) apply the PCR technique to confirm presumptive *Salmonella* colonies in food samples contaminated with *Proteus* swarming on selective isolation media, and (iii) evaluate the potential of interference of *Proteus* swarming on *Salmonella*-specific PCR band amplification.

MATERIALS AND METHODS

Microorganisms and samples. Eight ATCC strains from the National School of Biological Sciences-Instituto Politécnico Nacional (*S.* Typhimurium ATCC 14028, *Salmonella* ser. Choleraesuis ATCC 10708, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 19114, *Pseudomona aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633) and ten strains from the Central Laboratories of the Department of Health of the State of Jalisco-CEESLAB (*Salmonella* ser. Enteritidis, *Salmonella* ser. Poona, *Salmonella* ser. Gaminara, *Salmonella* ser. Michigan, *Salmonella* ser. Agona, *Salmonella* ser. Montevideo, *Proteus vulgaris*, *Proteus rettgeri*, *Vibrio cholerae*, *Shigella sonnei*), were used to determine the specificity of the PCR primers selected for this study. Food samples with presumptive *Salmonella* colonies were obtained from CEESLAB.

Evaluation of the specificity of selected PCR-primers with different bacterial strains. Before analysis of the food samples, two groups of PCR assays using the method described previously by Gutierrez, *et al.* 2002,[7] were carried out to verify the specificity of the primers to be used in this study. The first group: pure culture (10^8 CFU/ml) evaluation of eight *Salmonella* and ten non-*Salmonella* strains, and the second group: mixed culture (10^8 CFU/ml) evaluation of I) *S.* Typhimurium (positive control); II) *P. vulgaris* (negative control); III) *S.* Typhimurium and *P. vulgaris* mixture; IV) *S.* Typhimurium and *P. vulgaris* mixture incubated together for 24 h at 37° C. For each assay, 1 ml of dilution was centrifuged at 10,000 *g* at 4° C for 10 min. Genomic DNA was extracted using the rapid lysis method described by Laird *et al*.[10] The pellet was resuspended in 500 µl of lysis buffer containing 100 mM Tris-HCl (pH 8.5), 5 mM ethylenediamine tetra-acetic acid (EDTA), 0.2% sodium dodecyl sulphate (SDS; Sigma Chemical Co., St. Louis, MO), 200 mM NaCl and 200 µg/ml proteinase K (Invitrogen, Carlsbad CA, USA). Samples were incubated at 55° C for 75 min, cooled on ice, and the total DNA was precipitated by the addition of an equal volume of ice-cold isopropanol (Aldrich, Milwaukee WI, USA). Samples were stored at –20°C for 2 h. The DNA pellet was washed twice in 70% ethanol (Aldrich, Milwaukee WI, USA), air-dried and resuspended in 100 µl of DNAse-, RNAse-free distilled water (GIBCO, Invitrogen Corporation).

The PCR method was carried out using a set of specific primers that anneal to a region of the *hns* gene in *Salmonella* spp. and amplify a 152 bp fragment. The forward primer was 5' TAC CAA AGC TAA ACG CGC AGC T 3' and the reverse primer was 5' TGA TCA GGA AAT CTT CCA GTT GC 3'.[9] Amplification of bacterial DNA was standardized with 25 µl Ready-to-go™ PCR beads (2.5 units of *Taq* DNA polymerase; 10 mM tris-HCl; 50 mM KCl; 1.5 mM $MgCl_2$ and 200 µM of dNTP's; Amersham Bioscience, Piscataway NJ, USA), 0.8 µM of primers (Invitrogen, Carlsbad CA, USA), 4 µM of $MgCl_2$ and 5µl of DNA. PCR was carried out in a thermal cycler (Techne Progenie, Cambridge, UK) using the following conditions: initial DNA denaturation at 94º C for 5 min, 35 cycles at 92º C for 45 s; 60º C for 45 s; 72º C for 90 s and a final extension at 72º C for 10 min. The amplification products were also analyzed by electrophoresis at 90 volts, using an agarose gel (1.5%) stained with ethidium bromide (0.5 µg/ml). The gel was visualized with an UV transilluminator (UVP Upland, CA, USA) at 302 nm.

Microbiological analysis. Food samples were analyzed according to the microbial procedure described in NOM-114-SSA1-1994.[11] After the selective isolation stage, the plates with presumptive *Salmonella* colonies contaminated with visible swarming were selected (Fig 1). Although the XLD medium had turned to yellow in some areas, the colonies with black centers were evaluated as presumptive *Salmonella*, as swarming cells completely covered the plate surfaces. These presumptive *Salmonella* colonies contaminated with swarming were also analyzed by the biochemical confirmation procedure.

Identification of presumptive *Salmonella* colonies contaminated with *Proteus* swarming by PCR amplification. The presumptive colonies were resuspended in 1 ml of 1% sterile peptona water and incubated for 6 h at 37° C for PCR confirmation. These suspensions were harvested by centrifugation at 10,000*g* at 4° C for 10 min. Genomic DNA extraction, PCR amplification and electrophoresis of the presumptive *Salmonella* colonies were carried out under the same conditions used with the primer specificity assays.

Evaluation of *Salmonella* and swarming assays by PCR amplification. In order to determine the extent of *Proteus* swarming interference on PCR, specifically *Salmonella* band amplification, a test was carried out using artificial *Salmonel-*

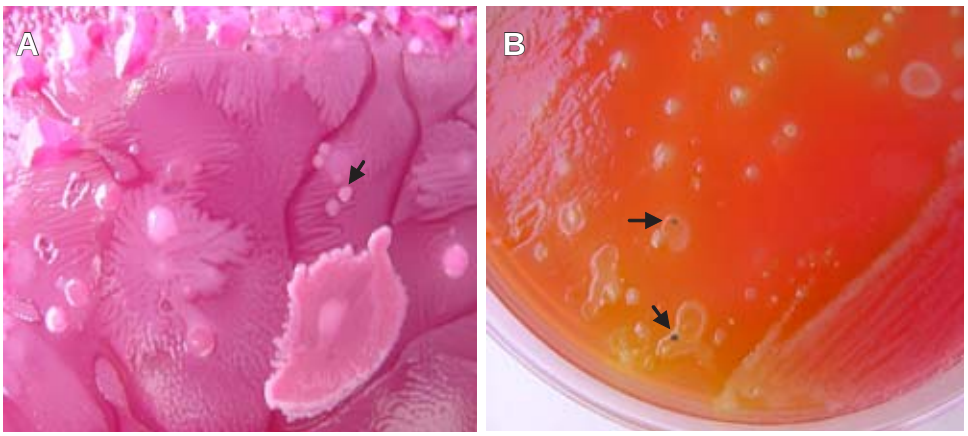

Figure 1. *Presumptive* Salmonella *colonies contaminated with* Proteus *swarming isolated from food samples. A) Brilliant Green Agar, B) Xylose Lysine Deoxycholate.*

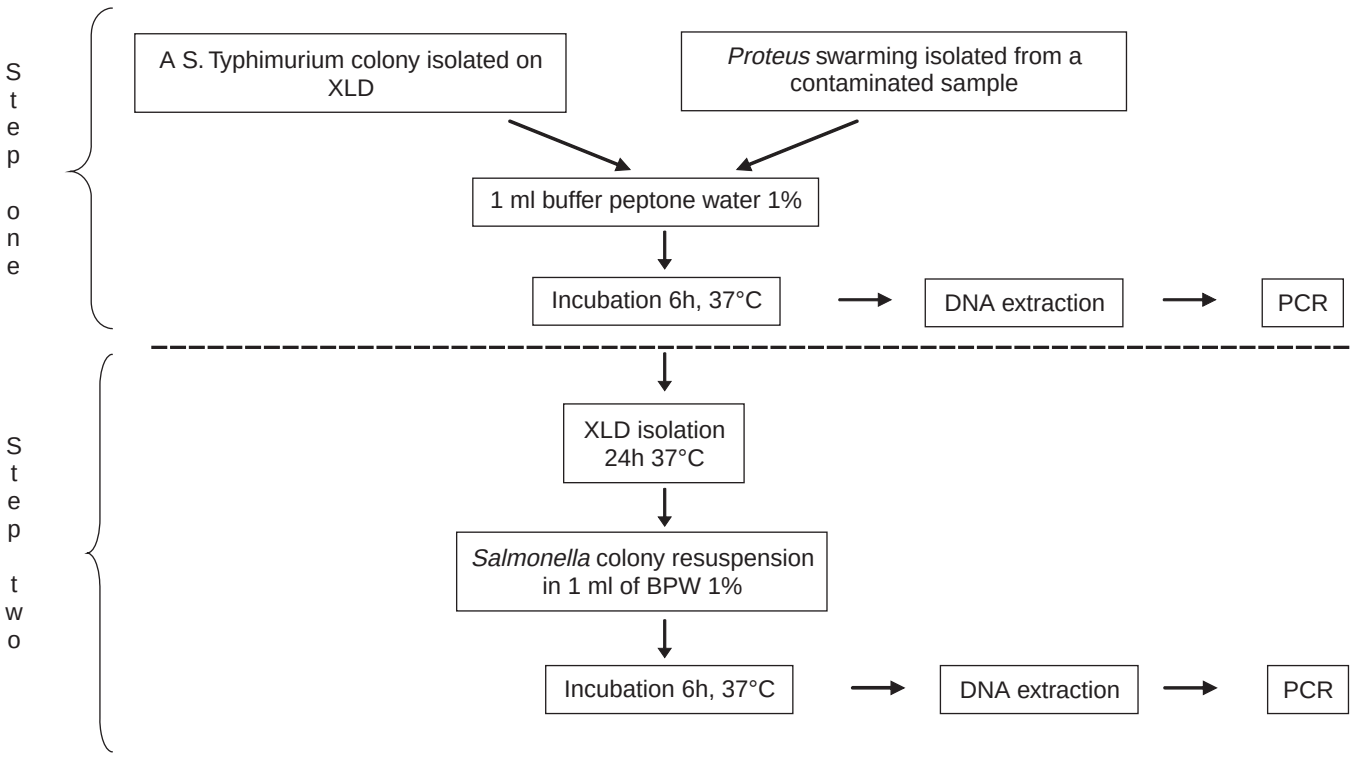

Figure 2. Salmonella *and swarming assays.*

la contamination with swarming in two steps. In step one, a *S.* Typhimurium colony (ATCC 14028) from a Xylose Lysine Deoxycholate medium (XLD agar, BIOXON, Becton Dickinson, Mexico) and a swarming isolate from an XLD plate were suspended in 1 ml of 1% sterile peptone water and incubated at 37° C for 6 h. This culture was centrifuged to obtain the bacterial pellet. DNA extraction and PCR amplification were achieved under the same conditions of the presumptive *Salmonella* samples. In step two, the *Salmonella* and swarming culture used in step one was isolated on XLD plates. After plate incubation at 37° C for 24h, a *Salmonella* colony contaminated with visible *Proteus* swarming was suspended in 1 ml of 1% sterile peptone water and incubated at 37° C for 6 h. This culture was centrifuged to obtain the bacterial pellet. DNA extraction, PCR amplification and electrophoresis were achieved under the same conditions as in the primer specificity assays. This assay was performed in the same way with Brilliant Green Agar plates (BGA, DIFCO Laboratories, Detroit Michigan, USA) as was used with XLD Agar. Both the BGA and XLD assays were carried out in duplicate (Fig. 2).

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RESULTS AND DISCUSSION

Evaluation of the specificity of selected PCR primers with different bacterial strains. The PCR amplification was performed using primers, which anneal to a region of the *hns* gene. The results generated an amplified 152pb fragment for all *Salmonella* tested. No amplification was detected when the specific primers were applied to non-*Salmonella* bacterial strains (Fig. 3). These results demonstrate that the selected primers are specific in the detection of *Salmonella* through PCR amplification.

Figure 4 shows the specificity results from mixed cultures of *Salmonella* and *Proteus*. Here, the 152pb *Salmonella* band is clearly shown in lanes 3 and 4, which indi-

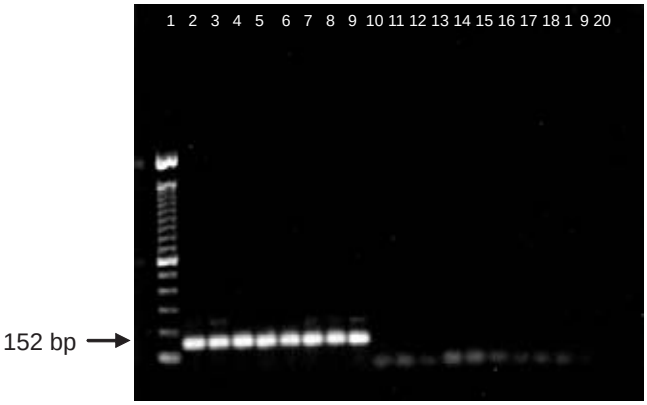

Figure 3. *Agarose gel electrophoresis of PCR products obtained using DNA extracted from pure cultures of 10^8 CFU/ml. 1) 100 bp DNA ladder, 2)* Salmonella *Typhimurium ATCC 14028, 3)* S. *Choleraesuis ATCC 10708, 4)* S. *Enteritidis, 5)* S. *Poona*, *6)* S. *Gaminara*, *7)* S. *Michigan*, *8)* S. *Agona*, *9)* S. *Montevideo, 10)* Proteus vulgaris*, 11)* Escherichia coli *ATCC 25922, 12)* Staphylococcus aureus *ATCC 6538, 13)* Listeria monocytogenes *ATCC 19114, 14)* Proteus rettgeri*, 15)* Vibrio cholerae*, 16)* Shigella sonnei*, 17)* Pseudomona aeruginosa *ATCC 9027, 18)* Bacillus cereus *ATCC 11778, 19)* Bacillus subtilis *ATCC 6633 and 20) negative control.*

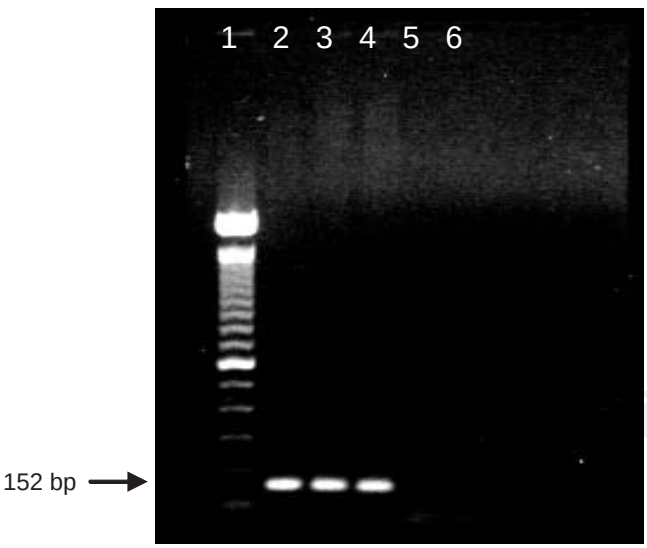

Figure 4. *Agarose gel electrophoresis of PCR products obtained using DNA extracted from mixed cultures of 10^8 CFU/ml. 1) 100 bp DNA ladder, 2)* S *Typhimurium (positive control) 3) Mixture of* S. *Typhimurium and* P. vulgaris *incubated together, 4) Mixture of* S. *Typhimurium and* P. vulgaris*, 5)* P. vulgaris *and 6) negative control*.

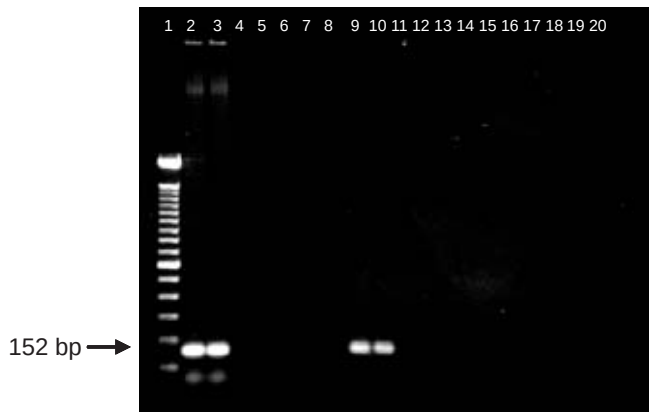

Figure 5. *Agarose gel electrophoresis of PCR products obtained from DNA extracted from presumptive* Salmonella *colonies contaminated with* Proteus *swarming from food samples. 1) 100 bp DNA ladder, 2)* S. *Typhimurium (positive control), 3)* S. *Typhimurium and* P. vulgaris*, 4)* P. vulgaris*, 5-19) presumptive* Salmonella *colonies contaminated with* Proteus *swarming and 20) negative control. Note: because the rest of the lanes of the colonies were negatives the figure with the other 50 samples results is not included in this article.*

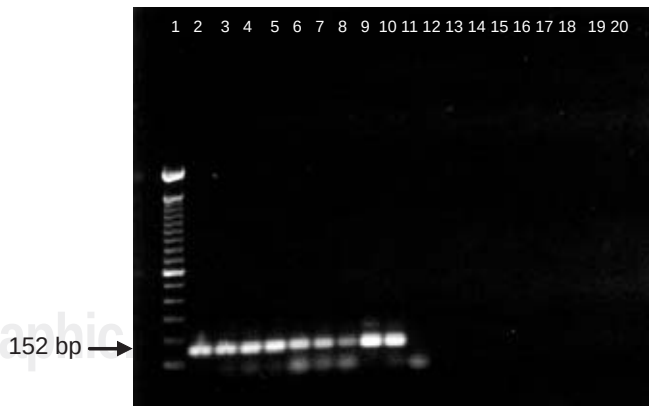

Figure 6. *Agarose gel electrophoresis of PCR products obtained from DNA extracted from mixed cultures of* Salmonella *and* Proteus *swarming. 1)100 bp DNA ladder, 2)* S. *Typhimurium (positive control), 3-4)* Salmonella *and* Proteus *swarming on XLD (first step), 5-6)* Salmonella *and* Proteus *swarming on BGA (first step), 7-8)* Salmonella *and* Proteus *swarming on XLD (second step), 9-10)* Salmonella *and* Proteus *swarming on BGA (second step), 11) negative control.*

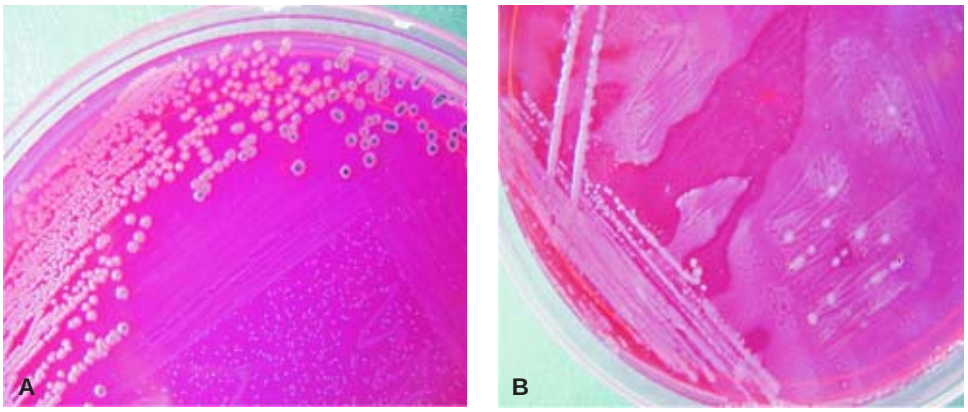

Figure 7. *XLD agar plate with mixed cultures of* Salmonella *and* Proteus *swarming. A)* S. *Typhimurium on XLD agar (positive control), B)* S. *Typhimurium contaminated artificially with* Proteus *swarming on XLD agar.*

cates that *P. vulgaris* does not cause any interference in the specific fragment amplification.

Microbiological analysis. The obtained results on biochemical confirmation of the 65 presumptive colonies were non *Salmonella* biochemical profiles for all the presumptive colonies.

Evaluation of presumptive *Salmonella* colonies contaminated with *Proteus* swarming by PCR amplification. Two of the 65 presumptive *Salmonella* isolates found in food samples were confirmed as *Salmonella* by PCR (Fig. 5). A positive result was indicated by the presence of the 152 bp *Salmonella* specific band in lanes 9 and 10. This result indicates that the PCR method used in this study may be successfully applied to confirm presumptive *Salmonella* colonies contaminated with *Proteus* swarming.

Evaluation of the swarming effect on *Salmonella* PCR amplification assays. As a result of the small number of positive *Salmonella* tests (two), additional assays of *S.* Typhimurium (ATCC 14028), artificially contaminated with *Proteus* swarming, were carried out to evaluate swarming interference by the PCR method.

PCR-amplification generated the 152 bp specific DNA band for all assays of *S*. Typhimurium artificially contaminated with swarming. These results demonstrate that swarming does not inhibit PCR-amplification (Fig. 6). In addition, it is important to note that when the assays of artificially contaminated *S*. Typhimurium were subcultured on selective plates (XLD and BGA Agar), a low number of *Salmonella* colonies were observed, despite of the use of a pure *Salmonella* strain. Finally, it was observed that after the 6h incubation period required to obtain the PCR sensitivity, the *Salmonella* colonies appearance changes in the solid media. This can be observed in fig. 7 were the *S*. Typhimurium colonies, confirmed as positives by PCR (Fig. 6), did not show the black precipitate.

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