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RAPID COUNTING OF PSEUDOMONAS FRAGI IN MEATS WITH AN ELISA METHOD

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INTRODUCTION

Over the last ten years, more and more works on rapid counting methods for micro-organisms have been published. This has been in response to the need for a more rapid evaluation of their concentration in processed foods. In highly perishable foods such as milk or meats, several techniques of rapid microbial counting have long been used with reasonable success. Although interesting results have been obtained with the ATP bioluminescence, or the radiometric methods, it should be emphasized that they only involve the dominant flora.

Serological methods provide very specific techniques for identifying micro-organisms. They have been used extensively to identify more or less pathogenic micro-organisms isolated from clinicals or foods in general. The enzyme linked immunosorbent assay (ELISA) is much more sensitive than most of the techniques used in classical serology. Its use is increasing rapidly in many laboratories. Spectacular results have been obtained recently to detect food toxins (PETERKIN and SHARPE, 1984). These findings have brought attention to the possible use of ELISA techniques for counting micro-organisms in foods, particularly the gram negative bacteria. As a matter of fact, it has been shown that many proteins of the outer membranes of these bacteria are common antigenic structures within a bacterial genus (HANCOCK et al, 1982). This has lead to demonstrate the possible use of the ELISA for counting Pseudomonas in chilled meats.

MATERIAL AND METHODS

1 - Bacteria

Seventeen strains of Pseudomonas isolated from meats were used in this study. They were isolated in our laboratory and were identified as Pseudomonas fragi according to the tests proposed by MOLIN and TERNSTROM (1983). Another strain (strain n° 13), was a gift of Pr. RICHARD (Institut Pasteur) it was used to produce antibodies against outer membrane proteins.

2 - Growth conditions and preparation of antiserum against O antigen

Bacteria were grown in Agar slants for 36 hours at 22°C in the chemically defined medium of EAGON and PHIBBS (1971). The Agar slants (4 tubes per each strain) were washed with 5 ml of saline and centrifuged for 15 min. at 10 000 r.p.m. The pellets were resuspended in 5 ml of saline and washed three times in the same conditions. The production of O antisera was made according to HANCOCK et al. (1982).

3 - Preparation of the outer membranes

After 36 hours of growth at 22°C in EAGON and PHIBBS (1971) medium the bacteria were collected and washed three times in saline. The cells (1.5 g wet weight) were treated according to the method of MIZUNO & KAGEYAMA (1979).

4 - Antiserum preparation against the outer membranes

The anti outer membrane proteins was done according to HANCOCK et al. (1982).

5 - Tube agglutination test

The O antigens (washed heated bacterial cells) were standardized to an optical density of 0.8 at 540 nm. Ten fold dilutions of serum in 0.2 ml volumes were made in 0.85 % saline. An equal volume of antigen was added. The tubes were then incubated overnight at 37°C followed by further incubation for 6 hr.s at + 4°C before reading.

6 - ELISA test

11 - Bacteria in pure culture

The OM prepared with the strain n° 13* (5 g of bacteria) were recentrifuged at 100 000 g for 1 h. The pellet was resuspended in 2 ml of 0.33 M Tris HCl pH 8.0 1 mM Na_2 EDTA buffer. This suspension was diluted to 1/100 with the carbonate-bicarbonate buffer pH 9.6 VOLLER et al. (1979) and pipetted (200 ul) into the wells of microtitration plates (FLOW Laboratories). After 18 h at 37°C, the coated plates were washed four times, with PBS tween, VOLLER et al. (1979) and shaken dry or stored at - 18°C. Bacteria were grown on Agar slants, (EAGON and PHIBBS (1971)) for 36 hr.s at 22°C. The fourteen strains were suspended in 33 mM Tris HCl pH 8.0 buffer so that a concentration of 10^7 bacteria/ml was obtained. A lysozyme plus EDTA solution was added in 0.5 % final concentration for the first and 1 mM for the second. The strains were kept 30 min. at 37°C. One tenth dilutions were made in carbonate-bicarbonate buffer pH 9.6 and 100 ul each were added to the wells of plates coated with the OM of the strain n° 13. 100 ul of a 1/200 dilution of the anti OM serum were then immediately added to the wells. The plates were incubated for 1.5 hr.s at 37°C and washed four times with PBS Tween. 200 ul of peroxydase coupled anti-rabbit IgG (0.5 %) were then added in each well and incubated 1.5 hr.s at 37°C. The plates were washed four times again with PBS tween and 200 ul of the substrate solution were added in each well (composition of the substrate solution : 100 ml of the phosphate citrate buffer pH 5.0, VOLLER et al. (1979), 40 mg of orthophenylene diamine 40 ul of 30 % H_2O_2). After 15 min. in the dark, 50 ul of 0.5 N sodium hydroxide were added. Test results were read with a multiskan spectrophotometer (Flow Laboratories) at 405 nm.

12 - Meat samples

Thirty one ground meat samples from a super-market were used. One tenth dilutions of the samples (3 g) were made in 33 mM Tris HCl pH 8.0 1mM EDTA Na_2 buffer. The lysozyme solution was also added in a 0,5 % final concentration. After 30 min. at 37°C, one tenth dilutions were made in the carbonate-bicarbonate buffer, pH 9.6. The following operations were quite similar to the ELISA test conducted with the pure cultures.

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*The strain n°13 was chosen because it does not cross react with the other strains, on the basis of the O antigen, and because its human origin would decrease the possibility of cross reactions with Pseudomonas isolated from meat which are different.

13 - Cross reactions of the Ps. 13 OM proteins with OM proteins of different gram negative bacteria

The bacteria used are :

Escherichia coli, Pseudomonas aeruginosa (PAO_1), Ps. fluorescens (n° 39), Ps. fluorescens (n° 13), Yersinia enterocolitica. All these strains have been taken from our laboratory collection. After an 48 hours culture at 10°C on the slope of nutrient agar tubes, the bacteria were washed with 2 ml of saline and centrifuged at 5 000 rpm for 15 min. The pellets (10^{10} bacteria) were diluted in a 33 mM Tris HCl pH 8.0, 1 mM EDTA buffer plus lysosyme 0.5 %. After 1 h at 37°C, decimal dilutions of the treated cells were made in the carbonate bicarbonate buffer pH 9.6 and 100 ul each were poured in microtitration plates (Flow laboratories). After 18 hours at + 4°C the plates were washed 4 times with PBS tween and 100 ul of the 1/200 dilution of the serum produced against the OM of the strain n° 13 were added. After 3 hours, the plates were washed with PBS tween and 100 ul of a 0.5 % dilution of the coupled anti rabbit IgG (GAR-peroxydase SERVA) were added. After 3 hours at 37°C and 4 washing with PBS tween, the plates were treated as previously described.

7 - Determination of viable counts

Immediately after the washing, and before adding the lysozyme solution, decimal dilutions were made in saline. The meat samples were similarly diluted after homogeneization with the same buffer. Diluted samples were poured into plates (LABADIE et al. (1975)). Plates were incubated 7 days at 23°C on a selective medium for Pseudomonas (FOURNAUD et al., 1973). That the colonies isolated from meat samples were Pseudomonas was confirmed according to SKINNER and LOVELOCK (1979).

RESULTS

1 - Cross agglutinations of the Pseudomonas strains

All the strains were agglutinated by their homologous serum. Antigenic homologies between several strains were noted. Antiserum raised against the n° 1 crossreacted with the n° 4 and the n° 5. The same observation was made with the serum raised against the n° 19, which reacted well with the n° 20. The other strains were only agglutinated by their homologous serum.

2 - Rapid Pseudomonas counting with ELISA

11 - Strains in pure culture

Figure 1 shows the antigenic homologies between the different Pseudomonas strains. The competition curves observed with the serum raised against the OM of strain n° 13 were quite similar, whatever the OM. This is in agreement with the fact that most of the proteins in the different OM are common antigens of Ps. fragi. The ELISA used in this study allowed us to detect 10^4 - 10^5 bacteria per ml in 4.5 h.

12 - Meat samples

Experimental countings of the Pseudomonas in meats with ELISA are very similar to the results observed in pure culture (figure 2). The samples which showed bad competition levels had low Pseudomonas concentrations. The relationship between the OD in ELISA and the counts seems linear from 10^4 bacteria/ml to 10^6/ml in our experiments (fig. 2).

13 - Cross reactions with different gram negative bacteria

No cross reactions were observed (figure 3) with the Enterobaciaceae used in this study. On the contrary the Pseudomonas strains cross react well with the OM proteins of the Pseudomonas n° 13, Ps. aeruginosa shows low cross reactions. The highest OD values are observed with Ps. n° 13 extracts, certainly because the anti OM serum contains anti O antibodies which are more antigenic than proteins.

DISCUSSION

Serological analysis of the Pseudomonas fragi O antigens has shown that most of the strains used shared few antigenic homologies. The very low number of strains studied certainly explains this. Grouping of Pseudomonas fragi on the basis of O antigens could be very interesting to show the most commonly found in meats. However, such a study, in order to have practical applications, must be done on many strains of different origins.

The isolation of Pseudomonas fragi OM allowed us to show that most of the proteins with this structure are probably common antigens as the competition curves established with all the strains are very similar. Moreover, many bands of identical molecular weights are systematically separated. This is in agreement with the results of NAKAJIMA et al. (1983) who showed that the electrophoresis of the OM within bacterial species could be used for taxonomical studies. More over, cross reactions with many gram negative bacteria commonly found in meats have only been observed with Pseudomonas species. This result show perfectly well that the ELISA proposed in this study could be used in meats as the main species isolated from meats are those which cross react perfectly well.

Results of the countings with ELISA allow us to show a relationship between the optical density observed with the ELISA test and the Pseudomonas counts. Thus, this technique could be suitable for rapid counting of Pseudomonas in meat products. However, before the general use of such a technique, many other test should be made on different meat products. In conclusion, ELISA for detecting or counting micro-organisms seems very useful since their advantages as regards specificit, safety and sensitivity have been well established. This prompts further work with well defined antigens, such as purified OM proteins, which could be used to detect Pseudomonas with more accuracy.

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