

1-1-2001

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Recommended Citation

YURDUSEV, NEVZAT (2001) "In vitro Model for the Study of Listeria and Salmonella Adherence to Intestinal Epithelial Cells," *Turkish Journal of Biology*: Vol. 25: No. 1, Article 3. Available at: <https://journals.tubitak.gov.tr/biology/vol25/iss1/3>

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In vitro Model for the Study of *Listeria* and *Salmonella* Adherence to Intestinal Epithelial Cells

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Received: 12.12.1998

Abstract : A quantitative test to study *Listeria* and *Salmonella* adherence to epithelial cells was developed. The quantitative test is rapid and allows the simultaneous testing of many variables such as the adherence ability of different bacteria to their target cells as well as the capability of various molecules to inhibit bacterial adherence. By using a strain-specific standard curve in each test, the test for the quantification of adherent bacteria became specifically sensitive. Non-viable biotinylated bacteria and immobilized cells or their extract were found relevant for the study of bacteria-epithelial cell interactions. Adherence ability of *Listeria* strains was found to be 10-fold that of the *Salmonella* strains used in this study. Bovine milk was able to inhibit at least 90% of the adherence ability of *Listeria* and *Salmonella* strains. Milk components may be useful for the identification of bacterial surface molecules involved in adherence, which is the first key step in the pathogenicity of invasive bacteria. Preliminary results indicate that the test may also be used to determine the competitive adherence ability of bacterial strains.

Key Words : Bacterial adhesion, *Listeria*, *Salmonella*, Epithelial cell, In vitro adherence model, Quantitative test, Adherence inhibition

Introduction

Adherence of pathogenic bacteria to host cells or tissues is a key step in virulence (1, 2). The complexity of in vivo adherence studies have been partly overcome by developing in vitro models (3-10). Adherence models use different combinations of bacteria and host cells: viable bacteria with living or immobilized host cells, or killed bacteria with either host cells in culture or animals (3, 8, 9, 11-14).

A number of potential bacterial or host cell surface molecules involved in the bacterial adherence to host cells have been determined by using different in vitro adherence test models (5, 15-20). As an in vitro model system for attachment, Sakellaris et al. (18) used CS1-mediated agglutination of bovine erythrocytes and CFA/I-mediated agglutination of human erythrocytes to determine the pilus tip protein involved in adherence of CS1 and CFA/I pili. The protein nature of the adhesive surface molecules of *Bacteroides fragilis* isolates (20) and the induction of the adherence ability of invasive bacteria following interactions between bacteria and epithelial host cells (11) have been also demonstrated by using in vitro assays.

Although several surface components of *Listeria* have been described as interacting with mammalian cells (21,22), major surface molecules involved in the adherence of *Listeria* strains to mammalian hosts cells have not yet been completely established. In contrast, it has been shown that bacterial viability was not essential for the adhesion of *L. monocytogenes* to the receptors on the host cell (21).

Thus, the development of an in vitro quantitative adherence model for the study of *Listeria* adherence seems necessary to identify major surface molecules involved in direct adherence to host cells. In order to study the direct adherence of *Listeria* to intestinal cells, in comparison with *Salmonella*, by using an epithelial cell-line (IPI-2I) of porcine ileal origin established by Kaeffer et al. (23), a quantitative in vitro test was developed. This test allows the quantification of adherent *Listeria* and *Salmonella* strains to non-viable whole IPI-2I cells or their extracts.

Materials and Methods

Bacteria: The strains of *Listeria* were *L. monocytogenes* (serotype 1/2a; ATCC 19111), *L. monocytogenes* (serotype 4b; ATCC 19115), *L. ivanovii* (serotype 5; ATCC 19119), *L. innocua* (serotype 6a; ATCC 33090), *L. welshimeri* (serotype 6a; CHUT 860477 (Centre Hospitalier Universitaire, Tours, France)) and *L. seeligeri* (serotype 1/2a; CHUT 860478). The strains of *Salmonella* were *S. choleraesuis* 33 and *S. abortus ovis* 15/5. Bacteria were grown on Tryptic Soy Agar (TSA) at 37°C. After harvesting, the bacteria was washed 3 times in sterile saline solution (NaCl 0.9%) (StSS).

Biotinylation of bacteria: Bacteria were killed by exposure to 0.05% peracetic acid and washed three times with StSS and labeled with N-hydroxysuccinimidobiotin (N-HSB, Sigma). Bacteria (1×10^{10} bacteria/ml) were incubated at 37°C in the dark with gentle agitation in N-HSB solution (300 µg/ml). After washing 4 times with StSS, they were adjusted to 2×10^9 bacteria/ml and kept in small aliquots at -20°C.

Establishment of standart curves of the biotinylated bacteria: Nine different concentrations of the biotinylated bacteria (from 1×10^4 to 1×10^8 bacteria/50 µl/well) were bound to ELISA plates (Nunc, Denmark) in 0.1M carbonate buffer (pH 9.6) for 2 h at 37°C followed by incubation with ethanol overnight at -20°C. After washing with PBS-Tween 20 (0.05%) (PBS-T), unoccupied sites of the plates were blocked with PBS-Bovine Serum Albumin (10mg/ml) (PBS-BSA). A streptavidin-horseradish peroxidase conjugate (Amersham Int., UK) (a dilution of 1:500 in PBS-BSA, 50ml/well) was added. After 25 minutes of incubation at 37°C, wells were washed 3 times with PBS-T and 3 times with PBS. Enzymatic reaction was developed by adding 50 µl/well of 0.1M citric acid buffer (pH 4.0) containing 0.88 mg/ml of 2,2'-azino-di (3-ethylbenzothiazoline sulfonic acid) (ABTS) and 8 µl/ml of 30% H₂O₂. After an incubation for 30 minutes at 37°C, the optical density (OD) was read at 414 nm in a micro-ELISA reader. Three

experiments were performed in duplicate. Non-biotinylated bacteria were used as negative controls. As an additional control, tests were performed without bacteria.

Cell culture: We used an epithelial cell line (IPI-2I) established from an adult histocompatible miniature boar (d/d haplotype) (18). This cell line is a subclone of a continuous ileal cell line immortalized by transfection with pSV3-neo, a plasmid containing the DNA sequences encoding large T and small t antigens of SV40. Cells were cultivated in DMEM (Gibco) containing 10% heated fetal calf serum, 4mM L-glutamine (Gibco), 0.024 IU/ml bovine insulin, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma).

Immobilization of IPI-2I cells and its SDS-extract: IPI-2I cells were freshly harvested and washed 3 times in sterile PBS. For adherence assay to whole cells, 3×10^4 cells in sterile PBS containing antibiotics were added to wells of sterile ELISA plates and incubated in a CO₂ incubator (5%) for 5 h at 37°C. Then, absolute ethanol (200 µl/well) was added and plates were kept at -20°C.

For adherence assay to cell extract, IPI-2I cells resuspended in 5ml of sterile PBS containing 2% sodium dodecyl sulphate (SDS), were agitated for 15 minutes at room temperature. After centrifugation (11000g, 30 minutes), the supernatant was harvested and an overnight ethanol precipitation was performed at -20°C. The supernatant, after centrifugation, was discarded and the pellet was solubilized in 1ml of 1% SDS-PBS. To fix the SDS-extract onto ELISA plates, 1:5 arbitrary dilution of the cell extract in carbonate buffer (0.1M, pH 9.6) was used. The plates were coated with 100 µl/well of the 5-fold diluted SDS-extract for 2 h at 37°C. The unoccupied spaces of the wells were blocked by either PBS-BSA (10 mg/ml) or PBS-skim bovine milk (10% v/w) (PBS-sM).

Adherence assay using immobilized cells and cell-extract: Fifty microliters of biotinylated bacterial suspension was added to each well, coated by IPI-2I and blocked by PBS-BSA. After 40 minutes incubation at 37°C, wells were washed 3 times with PBS-T. Adherent bacteria were detected by streptavidin-peroxidase conjugate and ABTS-H₂O₂ substrate as described above. Wells without immobilized cells or unlabeled bacteria were used as controls. For adherence assay to cell-extract, ELISA plates were coated with SDS-extract and used as described above for adherence assay with immobilized cells.

In order to determine whether a given bacterial strain is able to compete for adherence sites with another strain, identical experiments were performed with unlabeled bacteria. After incubation of the unlabeled bacteria, wells were washed 6 times with PBS-T and heterologous biotinylated bacteria was incubated at 37°C for 40 minutes and then washed 6 times with PBS-T. To verify the adherence of unlabeled strains, an immunoenzymatic detection was performed by monospecific mouse anti-sera to unlabeled bacteria (*L. monocytogenes* strains 19111 or 19115). Biotinylated goat anti-mouse (1:1000 in PBS-BSA, Jackson Lab.), streptavidin-peroxidase conjugate (1:500 in PBS-BSA, Amersham) and ABTS-H₂O₂ substrate were used for

immunodetection. After an incubation for 30 minutes at 37°C, the optical density (OD) was read at 414 nm in a micro-ELISA reader.

Results

In order to compare the adherence of 6 *Listeria* strains with that of 2 *Salmonella* strains, a quantitative adherence test was developed using killed bacteria and immobilized IPI-2I cells or their SDS-extract.

Establishment of quantitative adherence test: In the first step, to quantify the bacterial adherence, a specific quantitative standard curve for each biotinylated strain was established. The correlation between 9 different concentrations of biotinylated bacteria (1×10^4 to 1×10^8 /well) fixed onto the wells and the optical density (OD at 414nm) obtained with enzymatic reaction was studied for each strain.

No statistical difference in the OD values was observed between 1×10^7 , 5×10^7 and 1×10^8 biotinylated bacteria fixed onto the ELISA plate. These results indicate that the number of bacteria bound to the ELISA plate may be at the same level for these 3 bacterial concentrations (Fig. 1). In contrast, a very good linearity (coefficient of correlation, $r > 0.980$) was obtained with 4 bacterial concentrations (in \log_{10} , 5.0, 5.7, 6.0 and 6.7 for *Salmonella* and 5.7, 6.0, 6.7 and 7.0 bound bacteria/well for *Listeria* strains). Based on these results, these 4 biotinylated bacterial concentrations were used as the standard curve in each plate to determine the number of adherent bacteria.

Secondly, we determined the number of the labeled bacteria to be used as inoculum in the adherence assay. IPI-2I cells (3×10^4 cells/well) were immobilized onto ELISA plates. Then, the unoccupied sites were blocked by either PBS-BSA (10mg/ml) or PBS-sM (10% v/w). Different inocula of the biotinylated bacteria were freshly prepared in PBS-BSA or PBS-sM solutions and immediately added to the wells blocked with the identical blocking solution. The OD obtained (approximately 1.0 at 414nm) with an inoculum of 5×10^7 biotinylated bacteria has been found to be appropriate for quantitative adherence test (data not shown). From this result, 5×10^7 biotinylated *Listeria* and *Salmonella* strains/well were used as inoculum for all subsequent adherence assays.

Quantification of the adherence of *Listeria* and *Salmonella* strains: We studied the quantitative adherence of 6 *Listeria* and 2 *Salmonella* strains by using IPI-2I whole cells or its extract immobilized onto ELISA plates. The quantitative test showed that all of *Listeria* strains have an ability at least 10-fold that of *Salmonella* strains to both whole cell or its extract (Fig. 2). The number of adherent bacteria to the whole cell was found to be higher than that detected with the cell extract. A slight difference in the adherence ability of different *Listeria* strains had been observed. Non-statistical differences have been observed in bacterial adherence assays when PBS-BSA or PBS-sM were used as blocking solutions (data not shown).

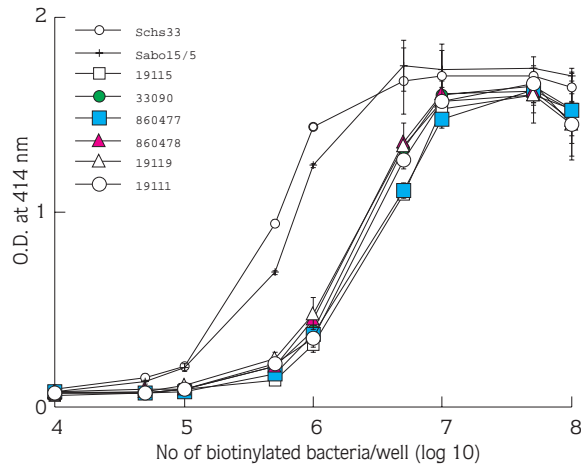


Figure 1. Establishment of standard curves for quantification of biotinylated bacteria.

Symbols are as follows: Schs: *S. choleraesuis* strain 33; Sabo15: *S. abortusovis* strain 15/5; 19111: *L. monocytogenes* ATCC 19111; 19115: *L. monocytogenes* ATCC 19115; 19119: *L. ivanovii* ATCC 19119; 33090: *L. innocua* ATCC 33090; 860477: *L. welshimeri* CHUT 860477; and 860478: *L. seeligeri* CHUT 860478.

Three experiments were performed in duplicate. Mean and SD of OD values were obtained from 6 experimental data.

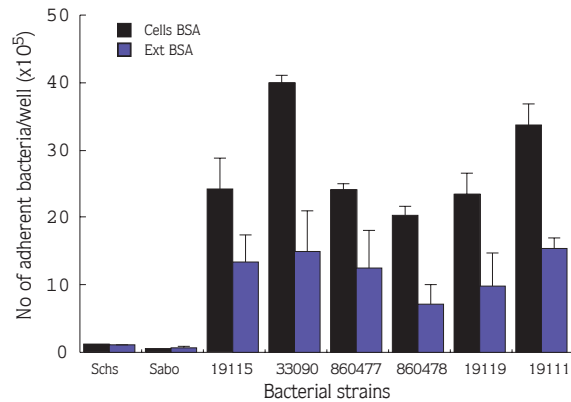


Figure 2. Adherence of biotinylated bacteria to whole cells (IPI-2I) and their SDS-extract.

The numbers of inoculated bacteria 5×10^7 bacteria/50 μ l/well for all strains. The abbreviations and identification number of strains were described in fig. 1.

Cells BSA: The immobilized IPI-2I cells were blocked by BSA.

Ext BSA: The SDS-extract of IPI-2I cells bound to ELISA plates were blocked by BSA.

Three experiments were performed in duplicate. Mean and SD of OD values were obtained from 6 experimental data.

These results suggest that the test is sufficiently sensitive and specific to enumerate adherent bacteria to the cell extract or to the target cell surface. The results demonstrate that it is possible to evaluate and quantify the adherence capability of bacteria by using killed bacteria and a non-viable target cell or its extract. Specific adherence of bacteria to both the IPI-2I cell or its extract indicates that complementary adhesive molecules of the target cell are also present in its SDS-soluble fraction.

Adherence inhibition by bovine milk: To test the effect of the milk on the bacterial adherence ability, bacteria were preincubated for 15 minutes at room temperature in either PBS-sM (10%) or PBS-BSA (10 mg/ml) blocking solutions and then washed with PBS. ELISA plates containing immobilized IPI-2I cells were blocked separately by the same blocking solutions. Fig. 3 shows that the bacterial adherence was strongly inhibited when bacteria were preincubated in the PBS-sM solution. However, when used as blocking solution, PBS-sM did not inhibit the bacterial adherence.

It was, however, important to know whether the milk components inhibit the binding of streptavidin to the biotin molecules covalently bound to bacterial surface. Therefore, biotinylated bacteria were fixed onto ELISA plates and incubated at 37°C for 2 hours with 3 different concentrations of PBS-sM (5, 10 and 20%) or PBS-BSA (10 mg/ml) for efficient saturation of the bound biotin. Non-blocked biotinylated bacteria were also used as controls. Fig. 4 shows that BSA and sM have no effect on the biotin-streptavidin detection system. Slightly higher OD

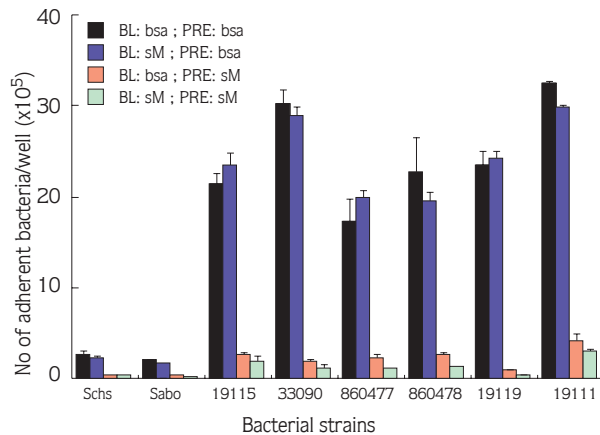


Figure 3. Inhibition of bacterial adherence to IPI-2I cells by bovine skim milk. The microwells onto which IPI-2I cells were immobilized were blocked by either BSA (10mg/ml) or sM (10%v/w). The biotinylated bacterial inocula (5×10^7 /50 μ l/well) were preincubated for 15 minutes with the blocking solutions before they were inoculated. BL: Blocking agent, PRE: Preincubation solution. Three experiments were performed in duplicate. Mean and SD of OD values were obtained from 6 experimental data.

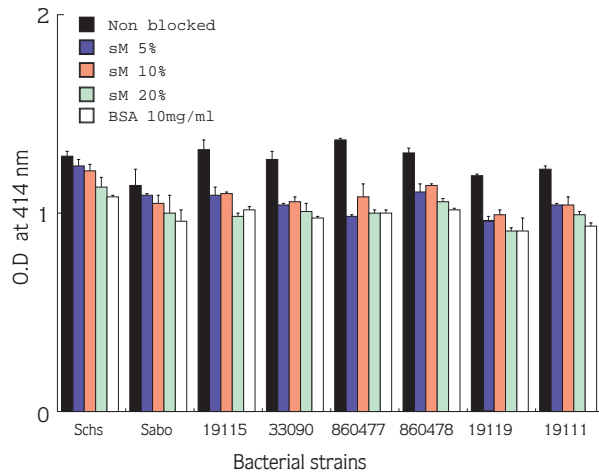


Figure 4. Effects of sM and BSA on the biotin-streptavidin detection system.

Biotinylated bacteria were preincubated in 3 different concentrations of sM (5, 10 and 20%) or BSA (10mg/ml) for 2 hours. Non-preincubated biotinylated bacteria (non blocked in figure legend) were also used as controls.

Three experiments were performed in duplicate. Mean and SD of OD values were obtained from 6 experimental data.

values observed with non-blocked bacterial strains were probably due to a non-specific binding of the streptavidin-peroxidase conjugate to non-blocked ELISA plates. These results suggest that the milk components do not inhibit the binding of streptavidin-peroxidase conjugate to biotin molecules covalently bound to bacteria. Thus, the skim bovine milk contains some components able to block adherent molecules present at the surface of *Listeria* and *Salmonella* strains.

Discussion

Listeria monocytogenes is a facultative intracellular pathogen whose initial site of infection is intestinal epithelial cells. The adhesion of pathogenic bacteria to the gut epithelium is the obligatory first step in the infection process. But in vivo *Listeria* adherence to host cells not well understood and the surface molecules of both *Listeria* strains and intestinal epithelial cells involved in the direct adherence process have not been well established. To overcome the complexity of the in vivo adherence process, a quantitative in vitro test to study the adherence of *Listeria* and *Salmonella* strains was developed.

In this test, non-viable *Listeria* and *Salmonella* strains were used to quantify the adherent bacteria to a non-viable IPI-2I cell-line (23). In a recent study, Maganti et al. (21) also clearly

demonstrated that bacterial viability was not essential for the adhesion of *L. monocytogenes* to the receptors on the host cell. The use of IPI-2I cells, a porcine origin intestinal epithelial cell line, is particularly relevant for adherence assay since intestinal epithelial cells have been found to be the initial site of infections caused by *Listeria* and *Salmonella* strains, and *L. monocytogenes* is also pathogenic for both human and porcine species (24-26). Moreover, viable IPI-2I cells are permissive to the penetration and multiplication of *S. choleraesuis* and *S. typhimurium* as well as *L. monocytogenes* (23, 27).

Previous studies have shown the similar ability of bacteria to adhere to both native and immobilized epithelial cells (8, 9, 12). Moreover, the relative percentage of viable *Listeria* strains adherent to the native IPI-2I cells in monolayer culture is similar to that obtained by non-viable immobilized cells and non-viable biotinylated bacteria (data not shown). The biotinylated bacteria used in this study are able to adhere not only to immobilized whole cells but also their SDS-extract (Figs. 2 and 3). Taken together, these data indicate that non-viable bacteria and immobilized whole IPI-2I cells or their extract retain the essential functional structures involved in direct adherence and can then be used in in vitro adherence tests allowing us to establish adherence ability of bacteria and to determine various components of both bacteria and host cells playing an essential part in the adhesion phenomenon.

The quantitative test is simple and rapid and allows us to quantify the bacterial adherence in 2 hours using immobilized cells or their SDS-extract. An important number of variables can be tested simultaneously. The minimal detectable number of biotinylated bacteria by enzymatic reaction is approximately 5×10^4 and 1×10^5 bacteria for *Salmonella* and *Listeria* strains, respectively. The detection system of the quantitative test is, therefore, sufficiently sensitive because the number of adherent bacteria detected in our experiments is approximately 10-fold the minimal detectable number of the biotinylated bacteria.

The use of a specific quantitative standard curve established for each biotinylated strain in each assay allows us to quantify adherent bacteria sensitively (Fig. 1). In addition, determination of the number of the biotinylated bacteria to be used as inoculum allows us to compare the quantitative adherence ability of different bacteria to IPI-2I cells and their extract. Standardization of these parameters renders our quantitative adherence test sensitively and specifically optimal for enumerating adherent bacteria to the cell extract or to the target cell surface. Another advantage of this test is the use of ELISA plates coated with immobilized IPI-2I cells after at least 2 months of storage at -20°C (data not shown).

In this study, it was demonstrated that skim bovine milk is able to strongly inhibit (>90%) bacterial adherence to host cells (Fig. 4). In preliminary experiments, a mixture of α -Lactose and β -Lactoglobuline was also found to be an inhibitor of *Listeria* adherence (data not shown). Adherence inhibition of *Listeria* strains observed in our preliminary experiments confirm and extend previous studies demonstrating the anti-adherence activity of human or bovine milk against adherent bacteria (8, 28, 29). Recent studies strongly suggest that some cell-surface

molecules such as internalin (*InIA*) and actin polymerization protein (*ActA*) (see: 30), *N*-acetylneuraminic acid (*NacNeu*) (21), a 55.3 kDa fibronectin-binding protein (22) and a 104 kDa protein (*p104*) (31) mediate, at least in part, the adhesion of *Listeria* spp. to host cells. In addition to these *Listeria* adhesive molecules, other surface molecules of different nature, such as those interacting with some components of bovine milk, may be involved in *Listeria* adherence.

Preliminary results indicate that unlabeled *L. monocytogenes* strain 19111 was able to inhibit the adherence of the biotinylated *L. monocytogenes* strain 19115 to the SDS-extract of IPI-2I cell. But unlabeled *L. monocytogenes* strain 19115 is not able to inhibit the adherence of the *L. monocytogenes* strain 19111 to the SDS-extract. Thus, these results indicate that these two *Listeria* strains may share the same adherence determinants, but *L. monocytogenes* strain 19111 possesses additional adhesive surface molecules. In a similar manner, Craven and Williams (32) have demonstrated efficient inhibition of the in vitro attachment of *Salmonella typhimurium* to cecal mucus by various strains of Enterobacteriaceae or Lactobacillus isolated from the intestines of adult chickens. The study of Craven and Williams (32) and our preliminary results indicate that in vitro competitive adherence assays have an important potential in the establishment of the competitive adherence ability of different bacterial strains and in the determination of both bacterial and host cell-surface molecules involved in adherence.

In conclusion, we developed an in vitro quantitative adherence assay for the study of interactions between bacteria and intestinal epithelial cell surface molecules and demonstrated that some components of bovine milk are strong inhibitors of the bacterial adherence. The results obtained in this study suggest that this adherence test may be useful for the determination of not only quantitative adherent capability of the pathogenic bacteria to various mammalian cells but also the complementary adhesive molecules present on the surface of various bacteria and target mammalian cells. This in vitro test may also help to rapidly identify different molecules having the ability to inhibit the adherence of bacteria to host cells or tissues, which is the first step in the infectious process of many intracellular bacterial pathogens.

Acknowledgement

The author acknowledges the scientific contributions of Dr. Philippe VELGE and Dr. Bertrand KAEFFER and the technical help of Elisabeth BOTTREAU in carrying this study. This study was carried out at Laboratoire de Pathologie Infectieuse et Immunologie, INRA, Nouzilly-France.

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