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Full Length Research Paper

Polyclonal antibodies production against *Staphylococcus aureus* protein A: ELISA technique optimization for milk quality control

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The main aim of this project is to produce polyclonal antibodies directed against the *Staphylococcus aureus* protein A and their use to appreciate bacteriological analysis of milk quality. In this context, an immunization produce was set up to test and detect in a batch of animals the convenient responder to the injected antigen. Furthermore, to optimize all parameters of retained ELISA test, a cross-table was conceived by using various concentrations of different reagents and has allowed selecting the optimal dilutions as follows: 1) 1/1000 for anti-*Staphylococcus aureus* protein A mouse antibodies and 1/2000 for anti-*Staphylococcus aureus* protein A rabbit antibodies. 2) 1/4000 for anti-*Staphylococcus* aureus treated by heat mouse antibodies and 1/500 for anti-*Staphylococcus aureus* protein A rabbit antibodies. 3) 1/2000 for anti-*Staphylococcus* aureus treated by NaClO mouse antibodies and 1/500 for anti-*Staphylococcus* aureus protein A rabbit antibodies. The application of optimized ELISA test to search and detect *Staphylococcus aureus* germs in different samples of milk has shown very satisfying results when compared to those obtained by bacteriological method. Indeed, the sensitiveness and the reproducibility, as well as the possibility to analyze a great number of samples in the same time at a reduced manner make the immunochemical method a best choice of test able to replace recent bacteriological methods.

Key words: Polyclonal antibodies, *Staphylococcus aureus*, protein A, ELISA, milk.

INTRODUCTION

The alimentary toxi infections are 300 to 350 times more numerous than indicated by the signaled cases (OMS, 1997). They result from the consumption of infected food by a noxious microorganism or a pathogenic agent able to produce toxins (Dacosta, 1995). The *Staphylococcus* is placed second in the rank of the bacterium responsible for alimentary intoxication after the Salmonellas (Derache, 1989).

In the case of the milk, the mastery of hygienic quality must satisfy the regulation norms imposed for the comercial exchange. Among the criteria usually considered contain the concentration in somatic cells, the whole flora, the presence and/or the importance of the contamination by the micro organisms causing a potential harm to the public health like *Staphylococcus aureus* (*S. aureus*) (JORA, 1994).

In spite of the recommendations inherent to the drastic hygienic measures, that readers and milk purveyors must follow, same manipulations can contaminate this product that becomes noxious for the consumer. Some thermic treatments permit to some stumps *S. aureus* to find their toxin genesis capacity (Hernandez et al., 1993). That is why the detection and the counting of *S. aureus* or of the *Staphylococuses* with a positive coagulase, keep their interest in alimentary microbiology and the enterotoxins staphylococcies dried dosage remains the unique means to define the food innocuousness.

The standard methods of cultivation used to detect

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Abbreviations: SpA, *Staphylococcus aureus* protein A; **ELISA,** Enzyme linked immunosorbent assay; **Sc,** *Staphylococcus aureus* treated by heat; **Sj,** *Staphylococcus aureus* treated by sodium hypochlorite NaClO; **As,** Anti-serum; UFC, unit forming colony.

microorganisms in the food are often laborious and time taking, as they need 3 to 7 days before decreeing if the dairy product is either good for the consumption or not. In the harmful food, these bacteria are often presented in (subletal) state and in a weak number when compared to the whole flora; this requires non selective enrichment steps for any bacteriologic approach. This method requires an expensive stock of big food quantities expecting the dosage results (OMS, 1997).

In the light of these considerations, the use of the immunochemical techniques in which analysis performances in the agro-alimentary domains are interesting. In fact to look for the presence of bacteria in the milk we adopt an immunization protocol. Those specific antibodies will be used then for an immunochemical dosage of enzyme linked immunosorbant assay (ELISA) type so as to identify or quantify the searched antigen. This realization is subjected to an appropriate immunization protocol, permitting in animal groups, to study and detect which responds better to the used antigen, once the antiserum is recovered and the optimal conditions of titling are defined permitting the application of the test ELISA in the identification of *S. aureus* in the milk.

MATERIALS AND METHODS

Preparation of the antigens

468.75 mg protein A, the principal constituent of the bacterial cell wall *S. aureus* (SpA) lyophilized (sigma lot 112 H 682) are made soluble in 2.5 ml phosphate plug 0.01 M, pH 7.4 containing NaCl 0.15 M (PBS).

A *S. aureus* solution (*S. aureus* 12600 of laboratories A.T.C.C. "American type culture collection" containing a bacterial charge of 10^5 UFC/ml is treated chemically or physically to constitute (to make up) the following samples:

1) *S. aureus* solution treated by heat (Sc) in 85°C during 10 min (Hernandez and al., 1993).
2) *S. aureus* solution treated by NaClO (Sj) in 8°C (Guiraud, 1998).

The two bacterial solutions saving Sc and Sj on a solid rank (Chapman) with streaks method followed by an incubation at 37°C during 24 h do not reveal any colony form which makes the treatments efficiency confirmed (Guiraud, 1998).

Immunization of the animals

Mice of stock Balb/c, females, 9 weeks old, provided by the Pasteur Institute of Algeriers are used. Seven groups of five mice are formed. The animals are immunized by intraperitoneal way. The animals of group 1 were at the SpA at 30 mg/ml. The ones of groups 2 and 3 were respectively the solution of *S. aureus* at 10^5 UFC/ml. This same solution is at half then at a quarter diluted. The animals of groups 5, 6 and 7 were immunized respectively by a *S. aureus* solution at 10^3 UFC/ml. The same solution is at a half then at a quarter diluted. So 1.25 ml of a adjuvant (complete for the first injection and incomplete for the recalls) are added to 1.25 ml to each antigenic solution. An aliquot of 400 µl is injected in the animal by intraperitoneal way. The protocol of immunization is the one recommended by Benali (1994).

Determination of the optimal titling condition by ELISA

Our choice is brought on a two times variant of ELISA method (Ternynck and Avrameas, 1991). The first step consists of forming complex antigen antibodies between the SpA and the mice antibodies and it takes place in a solid phase. In a second time this complex will be captured by the rabbit polyclonal antibodies anti SpA absorbed on a liquid phase and their content in mice immunoglobulin will be titled by goat antibodies anti-Ig of rabbit marked by the phosphatase alcalin. The enzymatic activity is measured with respect to a colorless substrate the para-nitrophenylphosphate (PNPP) that is transformed in colored para-nitrophénol (PNP) by hydrolysis which absorbance at 405 nm will be directly proportional to the antibodies.
The optimal proportions of each of the following reagents in the immuno-enzymatic dosage are systematically defined:

- Mouse polyclonal antibodies anti SpA.
- Mouse polyclonal antibodies of anti *S. aureus* killed by the heat (Sc).
- Mouse polyclonal antibodies of anti *S. aureus* killed by the NaClO (Sj).
- Rabbit antiserum anti SpA commercialized.
- *S. aureus* killed by the heat (Sc).
- *S. aureus* killed by the NaClO (Sj).
- Goat combined anti-Ig of rabbit marked with the alkaline phosphatase.

Microbiologic and immunoenzymatic search for SpA in the milk

The optimized ELISA test is applied to the SpA dosage in the milk. The infected sample of milk by *S. aureus* is provided by the industrial production milk staff of group (GIPLAIT) of Sidi Bel Abbes (west Algeria). A second sample of pasteurized and packaged milk is also analyzed.

Two analysis types, microbiologic and then immunoenzymatic were done. The microbiologic method consists of an enriching in the Giolitti and cantoni field a saving and isolation on Chapman agar-agar (Lebres and Mouffok, 1999).

The test with the coagulase and the one with the catalase were also carried out (Guiraud, 1989).
The immunoenzymatic analysis requires the preparation of an extract from milk. To minimize the non specific reactions of the test, the food extract must be limpid (Akhtar, 1998).

A polystyrene microtitling plate of 96 shafts (Nunc) was used. The shafts from A2 to H2, A6 to H6 and A10 to H10 were doubly presented by respectively A1 to H1, A5 to H5 and A9 to H9 and were reserved as witnesses.

The shafts from A4 to E4, A8 to E8 and A12 to E12 are doubly presented by respectively A3 to E3, A7 to E7 and A11 to E11 and are reserved for the standard.

The shafts A3, A7 and A11 represent the positive controls (a head solution milk standard is used).
The shafts B3, B7 and B11 represent the negative controls. The milk solution was replaced by the saturation buffer of the absorbant sites PBS-Tween-Gelatin-Polyvinylpyrrolidone (PBS-T-G-PVP) (Towbin and Gordon, 1986).

The shafts C3, C7 and C11 represent the witness of the mice antiserum (the mice antiserum was replaced by the buffer PBS-T-G-PVP).

The shafts D3, D7 and D11 represent the witness of the rabbit antiserum (the rabbit antiserum was replaced by the buffer PBS-T-G-PVP).

The shafts E3, E7 and E11 represent the witness of the conjugated (a conjugate was replaced by the buffer PBS.T-G-PVP).

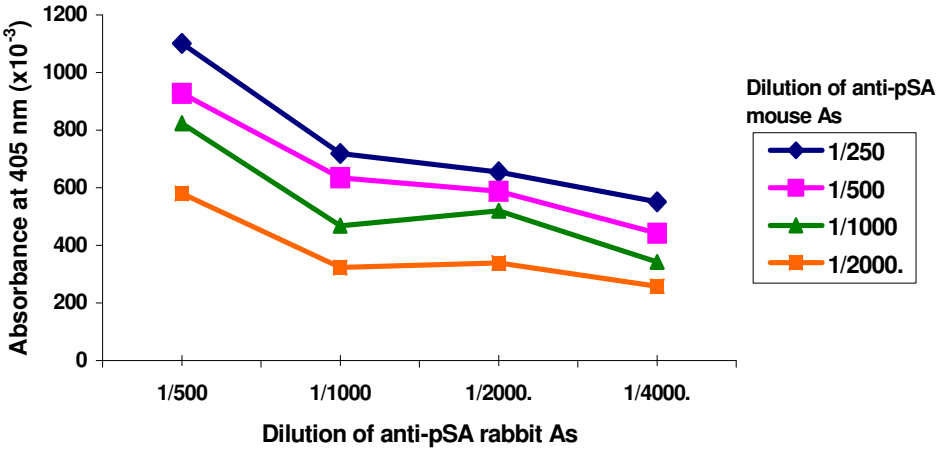

Figure 1. Variation of the dilution of anti-SpA mouse As according to that of rabbit As at 1.25 µg/ml of SpA.

The shafts of columns 1, 2, 3 and 4 were sensibilized by mice antiserum anti SpA diluted at 1/1000, the ones of columns 5, 6, 7 and 8 by a mice antiserum anti-Sc at 1/4000 and the ones from columns 9, 10, 11 and 12 by mouse polyclonal anti Sj diluted at 1/2000.

Eight decreasing dilutions at 2 (1/2 to 1/256) were prepared from the head solution standard of a milk extract in a dilution buffer (PBS-T-G-PPV).

100 µl from each dilution of antigenic solution were introduced in the shafts sensitized and represented as double of A1 (A2) to H1 (H2), A5 (A6) to H5 (H6) and A9 (A10) to H9 (H10).

The rabbit anti serum anti SpA was introduced in a dilution of 1/2000 in the shafts of columns 1, 2, 3 and 4 and at 1/500 in the shafts of the eight columns left.

The standard curve was realized by a series of dilution at 2 (1/2 at 1/256) from a head solution of SpA at 5 µl (Paraf and Peltre, 1992).

Each solution was made to react with 3 types of mice antiserum and with the one of the rabbit anti SpA.

RESULTS AND DISCUSSION

Determination of the optimal conditions of titling by ELISA

The experimentation is led under a series of crossed board forms. The rabbit immunoglobulins were detected by a dilution fixed at 1/30000 of a goat Ig anti-rabbit Ig marked with the alkaline phosphatase.

The concentration in PNPP substrate is fixed at 1 mg/ml (Ternynck and Avrameas, 1991). The ELISA test adopted gives a variation coefficient intra-assay not overcoming 6%. Despite the variation of the optical density, it is significant when it is upper or equal to the double of the one witnessed at without antigen (Metenier et. al., 1987).

The mice antisera taken at the 4th and 54th immunization day which their concentration with antibodies was important were used. This study allowed us to define the different reagent dilutions used, permitting the optimization of the ELISA test.

Figure 1 shows the influence of the dilution rate of the mice antiserum anti-SpA on the curve of optical densities according to the dilution rate of the rabbit antiserum anti-SpA, for a fixed concentration in SpA of 1.25 µg/ml. Also the mice antisera anti-SpA were, respectively, of 1/1000 and 1/2000 at 1.25 µg/ml of SpA.

Comparing the different dilutions of the polyclonal used, we notice that the dilution 1/500 of the mouse polyclonal anti-*S. aureus* killed either the NaClO (Figure 2) or by the heat (Figure 3) and 1/2000 of the rabbit antisera anti-SpA were optimized dilutions kept for the dosage of the SpA by antiserum directed initially against the *S. aureus.*

Identification and qualification of the SpA in the milk

Microbiological analysis

1st sample: The incubation of the sample of milk diluted to 1/10 at 37°C during 24 h in the Giollitti Cantoni blackened field. However, after isolating on a Chapman field, mean size colonies, and elaborating some pigments, a yellow color appeared. The mixture of the stumps with the rabbit plasma reveals the presence of a clot. The gaseous freeing was seen whenever the cultivation was kept in touch with hydrogen peroxide which confirmed the presence of the catalase. Most of these tests make the contamination of the 1st sample by *S. aureus* confirmed.

2nd sample: This milk sample at a dilution at 1/10 did not reveal any change after incubation at 37°C during 24 h in the Giolitti cantoni field; which confirmed the absence of *S. aureus*.

Immunoenzymatic analysis

The using of the different mice anti-sera for the coating,

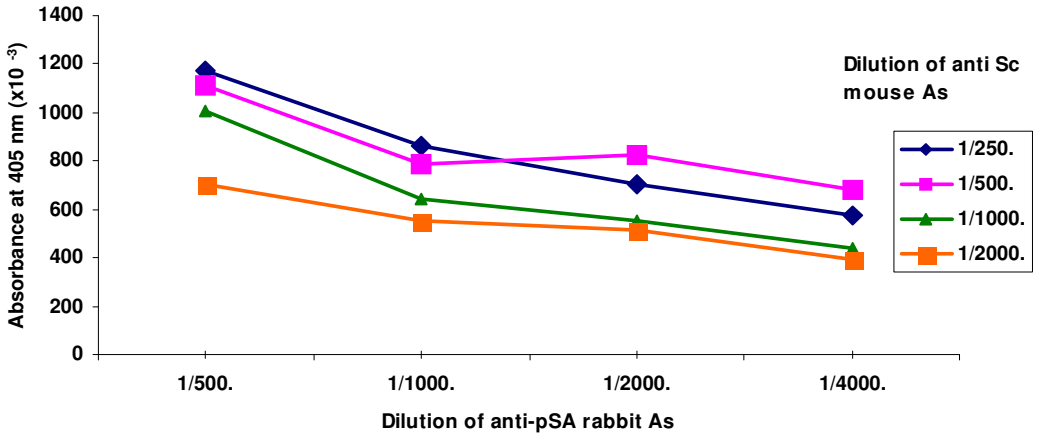

Figure 2. Variation of the dilution of anti-Sj mouse As according to that of rabbit As at 1.25 µg/ml of SpA.

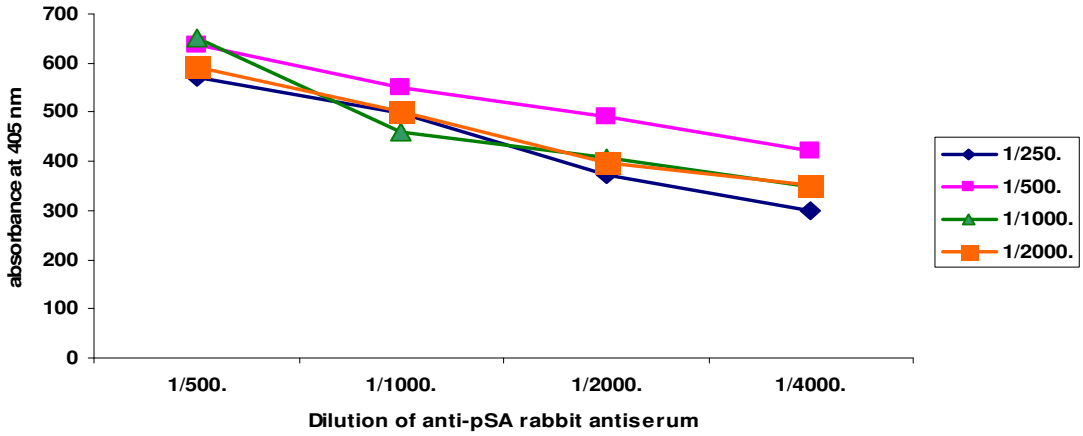

Figure 3. Variation of the dilution of anti-Sc mouse As according to that of rabbit As at 1.25 µg/ml of SpA.

has given higher optical densities ranging from 0.882 (Figure 5) to 1.446 (Figure 6) in the case of the infected milk and weak optical densities ranging from 0.174 (Figure 4) to 0.3999 (Figure 5) in the case of the uninfected milk. These results corroborate those obtained by the microbiological method.

The ELISA test permits to quantify the SpA in the two milk samples. A standard curve that showed that the optical densities are not significant beyond a dilution at 1/4.

The Figures 4, 5 and 6 show that the infected milk sample contained 2.5 to 5µg/ml of SpA. This protein was absent in the 2nd sample.

The optical densities obtained when analyzing the 1st sample of infected milk, by using the three types of antisera anti-SpA, anti-Sc and anti-Sj are significant only when the extract of the analyzed milk is used without dilution.

The Figure 4 represents the results of the Immunoenzymatic analysis of the two milk samples in the case of the use of the mice antiserum anti-SpA for the coating. The results show that the mice antiserum anti-SpA permit to detect the presence of this protein in the milk sample which is said to be infected according to the microbiologic analysis. With an absorbance of 0.730, the standard curve permits an estimation of this protein rate in the milk to 3.4 kg/ml. The same anti serum did not reveal any trace of SpA in the 2nd sample (safe milk) as the optical density remains very weak (0.174).

The Figure 5 expresses the results of the milk immunoenzymatic analysis using a nice anti-serum anti Sc in the first sensibilisation step. The use of this type of antiserum revealed the presence of the *S. aureus* in the first milk sample with a high enough absorbance (0.882). The use of this antiserum anti Sc showed the absence of these microorganisms in the milk and confirmed its innocuousness (DO = 0.399).

The results of the immunoenzymatic analysis of the milk using the nice antiserum anti *S. aureus* killed by the NaClO for the coating are represented in the Figure 6. The results of the microbiological analysis are confirmed by the immunoenzymatic analysis appreciations as the

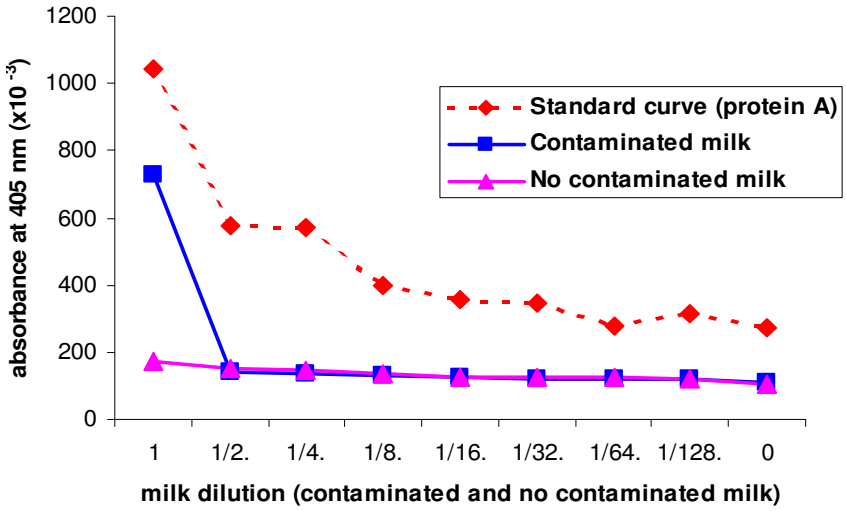

Figure 4. Immunoenzymatic analysis of two sample of milk (safe and infected) with utilization of anti-SpA mouse As for the coating.

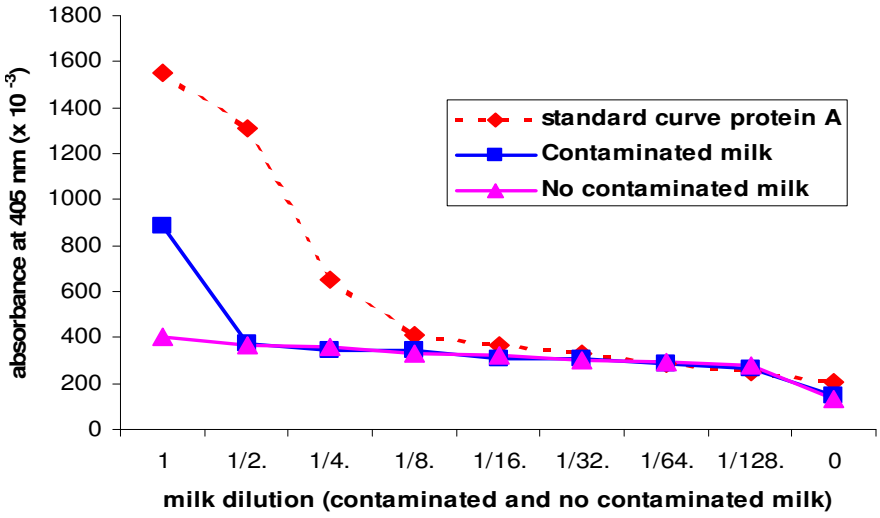

Figure 5. Immunoenzymatic analysis of two sample of milk (safe and infected) with utilization of anti-Sc mouse As for the coating.

mice antiserum anti-Sj detects the presence of the *S. aureus* in the milk (DO = 1.146) and consequently the presence of the SpA. The same antiserum did not reveal a contamination of the 2nd milk sample (DO = 0.386).

The three types of mice anti-sera anti SpA and Sj used for the coating permit the identification of the SpA but the first remains the antiserum of choice.

Conclusion

The immunoenzymatic dosage results turned out to be similar to the ones of the microbiological analysis. The three antiserum types used have detected the SpA presence in the milk is said to be infected sample according to the microbiological analysis and have also confirmed its contamination by *S. aureus*. However, the same polyclonal have detected no trace of SpA in the sample of the safe milk as well as its innocuousness.

The immunoenzymatic analysis has a big income if compared to microbiological analysis. It permitted us to define the SpA rate in the infected milk sample. By using a standard curve of this protein, we could estimate its quantity (3.4 mg/ml). The guided antiserum against the SpA seems efficient for the *S. aureus* search in the food.

The optimized ELISA test is of an outstanding sensitiveness. The reproducibility as well as the analysis possibility of a great number of samples was the same and reduced the time of this immunochemical method a choice method able to replace the microbiologic classical

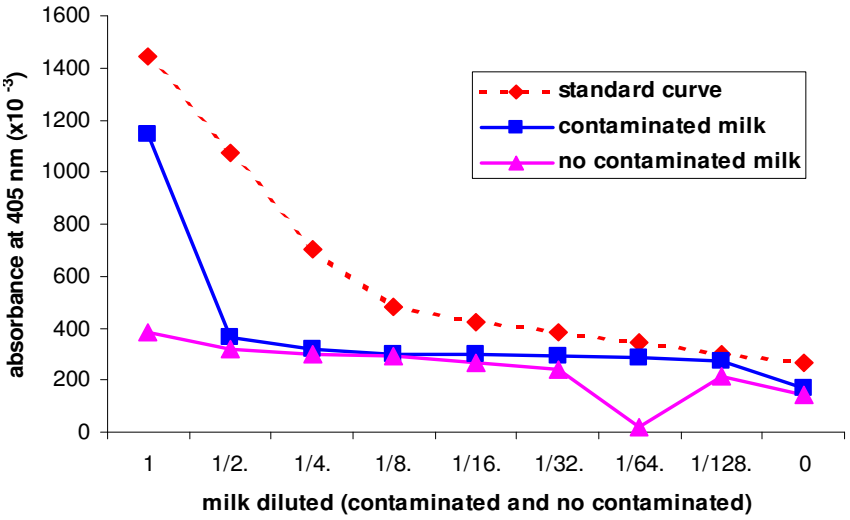

Figure 6. Immunoenzymatic analysis of two sample of milk (safe and infected) with utilization of anti-Sj mouse As for the coating.

methods currently used.

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