CHAPTER 22

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN IN MUSHROOMS

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1. INTRODUCTION

The detection of the staphylococcal enterotoxins (SE) in foods is a subject that has attracted wide attention and has resulted in research in a number of countries. There are two main reasons for this interest: 1) to determine whether a particular food was involved in food poisoning; and 2) whether a processed food suspected of possible contamination is marketable. Detection of enterotoxin in a food implicated in food poisoning is normally easier than establishing the safety of a food because in most instances the amount of enterotoxin present in the foods involved in food poisoning outbreaks is much more than the minimum amount necessary to establish the safety of a food.

2. DEVELOPMENT OF DETECTION METHODS

It was not until the first enterotoxin was purified (Bergdoll *et al.*, 1959b) and specific antibodies to it were prepared (Bergdoll *et al.*, 1959a) that it was possible to develop laboratory methods for its detection. The fact that the enterotoxin was a protein made impossible the development of chemical methods for its detection as such methods were not specific for any given protein. As it turned out, only methods that involved the reaction of the specific antibody with the enterotoxin were useful in its detection. During the course of purifying the first enterotoxin, gel diffusion methods based on antigen-antibody reactions to produce a precipitate were being developed. Such methods as the single gel diffusion (Oudin, 1952) and double gel diffusion tube methods (Oakley & Fulthorpe, 1953), the plate methods (Ouchterlony, 1948), and the microslide (Crowle, 1961) were among those presented. The Ouchterlony plate method was the method used initially to check culture supernatant fluids for enterotoxin because of its simplicity (Robbins *et al.*, 1974). However, its sensitivity was not such that it could be used to detect enterotoxin in food extracts from suspect foods.

Dr. Ezra Casman at the United States Food and Drug Association (FDA) also was working on the staphylococcal food poisoning problem. The method that he and Mr. Reginald Bennett (1965) developed required the concentration of the food extract from 100 g of food to 0.1 or 0.2 ml with the

final analysis by microslide. The microslide is the most sensitive of the gel diffusion methods, but not an easy method to use. The first use of this method was in the testing of cheese that had been implicated in food poisoning (Zehren & Zehren, 1968). At that time, the limit of sensitivity appeared to be about 1 µg/ml but, with later improvements, some individuals were able to achieve a sensitivity of 0.1 µg/ml (Casman *et al.*, 1969). However, most users of this method are unable to detect less than 0.5 µg/ml. The maximum sensitivity reportedly achieved is 30-50 ng/ml, but this would be a rare occurrence. It is doubtful that any individual using the extraction-concentration microslide method would be able to detect less than 1 ng of enterotoxin/ml and for most individuals, 1 ng/ml would be impossible. One of the problems with this method is that the percentage of enterotoxin recoverable, particularly at the very low levels, is probably not more than 10% and may be much less. Although this method is referred to as the "Official Method" for detection of enterotoxin in foods (Bennett & McClure, 1980), the fact that most individuals cannot achieve the maximum sensitivity claimed for it discredits the method. Thus, this method is not one to be recommended for use in the detection of enterotoxin in foods, especially in the establishment of the safety of foods.

3. SENSITIVE DETECTION METHODS

The quantity of enterotoxin required to cause illness in sensitive individuals was estimated to be less than 1 µg from analysis of foods involved in food poisoning (Bergdoll, 1979). It was only an estimate because enterotoxin is not uniformly distributed in foods nor is the amount of food consumed by any one individual known. It was not until an outbreak among school children who had drunk a known quantity of chocolate milk could a more accurate estimate be made. The amount present in the amount of milk drunk was 144 ng, or 0.4 to 0.75 ng/ml of milk, which was barely detectable by enzyme-linked immunosorbent assay (ELISA) without some concentration of the milk (Evenson *et al.*, 1988). It should be pointed out that the FDA laboratories were unable to detect enterotoxin in the milk served on the day of the outbreak (Schwab, personal communication). In most food poisoning outbreaks, much more enterotoxin is present and is easily detectable. However, it is essential that methods be available to detect less than 0.5 ng/g of food, to declare a suspect food consumable.

Fortunately, sensitive methods for detection of enterotoxin are now available, such as radioimmunoassay (RIA) (Miller *et al.*, 1976), ELISA (Freed *et al.*, 1982), and reversed passive latex agglutination (Igarashi *et al.*, 1985), These require no concentration of the food extract to detect ≤0.5 ng enterotoxin/g food although, if necessary, the extract can be concentrated several fold without interfering with the analysis. Although these sensitive methods have been available for a number of years and even though personnel in the FDA laboratories developed satisfactory procedures for both RIA (Kaufman, personal communication) and ELISA (Kaufman, 1980), FDA still insisted on using the cumbersome and outdated extraction-concentration microslide method. The most disturbing aspect of this was that, as a result of a collaborative study on the use of this method, it was declared the "Official Method" for the detection of enterotoxin in foods (Bennett & McClure, 1980). This was done without the involvement of the Bergdoll laboratory, the leading group in the development of methods for the detection of enterotoxin in foods. It was only after the paper had been published that Bergdoll was aware of the collaborative study.

As a result of the publication, Bergdoll wrote to FDA about the acceptance of the collaborative study as adequate for it being made the "Official Method". One paragraph from his letter read as follows: "The method presented in the article does not agree with the one presented in the 'Compendium of Methods for the Microbiological Examination of Foods' (Bergdoll & Bennett,

1984). The amount of food used in the collaborative study was 20 g instead of 100 g and the amount of toxin added was 0.1 µg to 0.2 µg per gram of food which is much higher than is recommended in the Compendium (0.1 to 0.25 µg/100 g of food, 2nd Edition, p 388). It is recognized that the extraction-concentration-microslide method is not an easy method and many people have difficulty obtaining the sensitivity possible, but this does not justify changing the sensitivity required and making the extraction easier to get good results in a collaborative study. Even so, only 73% were able to obtain positive results with the samples to which toxin was added. The fact that 99% were able to identify negative samples is of less importance." It is questionable whether there should be an official method for the detection of enterotoxin in foods. The basic reaction is immunological instead of chemical and is not easily quantifiable. Some individuals will find one procedure better for them than others, and they should be allowed to use that method. The only stipulation should be that a minimal amount be detectable. Certainly, when better methods are developed, they should replace the older methods. It is difficult to understand why FDA insists on using a long outdated method in favor of methods which are more sensitive and easier to use.

4. SCREENING KITS

Kits are now available commercially for the detection of enterotoxin in foods. The first one available was the ELISA ball kit produced in Bern, Switzerland (Fey & Pfister, 1983). In this kit, the specific antibodies are adsorbed to the surface of polystyrene balls, each color coded for a particular enterotoxin. One ball for each SE (SEA, SEB, SEC, SED) and two control balls coated with normal rabbit sera are added to 20 ml of the food extract and shaken over night to allow the enterotoxin to attach to the specific antibodies. The value of using this volume of extract results in increased sensitivity. The conjugate is alkaline phosphatase attached to the specific antibodies. The substrate is nitrophosphate which is converted to a yellow compound by the phosphatase. The sensitivity of the method can be as high as 0.1 ng/ml. In tests to date, it appears to be the method of choice (Wieneke & Gilbert, 1987; Wieneke, 1991).

The RPLA kit was developed in Japan and employs latex particles coated with the specific antibodies for SEA-SED (Igarashi *et al.*, 1986; Fujikawa & Igarashi, 1988). The treatment of the coated particles with SE results in agglutination of the latex particles. There are at least two problems with this method: 1) it is necessary in many instances to filter the food extract through a non-protein adsorbing filter to clarify the extract, and 2) an occasional food extract will give a false agglutination (Wieneke & Gilbert, 1987). The sensitivity of the method appears to be somewhat less than the ELISA ball kit (Wieneke & Gilbert, 1987).

More recently, screening kits have been developed which contain all of the specific antibodies adsorbed to the same surface such as wells in a microtiter plate. The first one marketed was the TECHRA kit developed in Australia and promoted by FDA. One problem with screening kits is obtaining an equal quantity of specific antibodies for each SE to each surface. Apparently this is very difficult since, in the TECRA kit, the analysis for SEB is twice as sensitive as that for the other SEs. Tests on at least one kit in Japan showed very poor results with SED (Igarashi, personal communication). Nonspecific reactions have been encountered (Park *et al.*, 1992).

A second screening kit has been produced, that by TRANSIA in Lyon, France. In this kit the inside bottom of small tubes is coated with the specific antibodies. The sensitivity is equal to 0.02 ng of SE; a mixture of 0.004 ng of each of the five SE gives a beautiful blue color. A collaborative study with this kit has been done and a paper was submitted to the AOAC journal

(De Solan *et al.*, 1992)

The screening kit idea is a good one providing all SEs can be detected equally. The advantage is that if more than one SE is present, and present at below the individual detectable levels, it is possible that together they could be detected. This is important because it is known that the biological reaction of the SEs is accumulative (unpublished data).

A procedure included in the TECRA kit that has caused a lot of concern is the use of urea to supposedly renature heat-treated SEs. The exact effect of heat on proteins is not certain but, from experience, the structure is so altered that they are no longer active, for example, enzymes. The idea that heated SE can be regenerated is fascinating, but unproved. It is known that urea can partially unfold the SE molecule and treatment with guanidine hydrochloride results in complete unfolding (unpublished results). However, removal of the denaturing agent allows the molecule to refold to its original structure and retain its biological activity. Denaturation by heat is quite different and it is very doubtful that heated proteins can be renatured by urea. Even so, it is known that the heat-denatured SEs are not biologically active when taken intragastrically. This has been demonstrated by boiling the SE over a 30 minute heating period at 100°C. It is true that the results reported by Dangerfield with heated SEA appear to show that the activity is enhanced by the heating procedure (Dangerfield, 1973). This was never confirmed and remains questionable, especially in the light of the experiments carried out at the National Canners Association (Denny *et al.*, 1966, 1971; Humber *et al.*, 1975). The time required to inactivate SEA by heating in beef boullion decreased exponentially with the increase in temperature. The activity was followed by intragastric injection into monkeys and intrapertioneal injection in cats. The SE could be detected serologically even though it was no longer active in monkeys. This is not unexpected because the action in monkeys is not very sensitive. The important point is that SEA was definitely inactivated with time.

There is one curious aspect to the procedure employed in the treatment with urea. The food extract treated with urea is concentrated 20-fold, and the result compared to the unconcentrated extract. It would be presumed that one should be able to detect any SE that was present in too small amount to be detected in the unconcentrated extract.

If more SE were detectable after the urea treatment, it could be due to one or two other phenomena. It is known that, on relative mild heating, the SE can associate with food proteins and not be detectable when the usual extraction is done (Satterlee & Kraft, 1969). In this case, any SE adsorbed would be disassociated with urea and could be detectable. The other phenomenon is that the SE may dimerize and could be dedimerized with urea, thus increasing the amount of SE detectable. In either case, the SE would not be denatured and would still be biologically active. In the case of the mushrooms, it would be expected that the retorting would result in denaturation of the SE, and it would be biologically inactive.

The 20-fold concentration step raises the question of how sensitive do the methods need to be. If such a step is desirable, it would complicate the SE analysis because it would make it more difficult as a means of concentration would be needed. The sensitive methods now in use made it possible to eliminate the cumbersome concentration procedures. It is hoped it would not be necessary to revert to concentration procedures.

5. CASE STUDY

The mushroom problem arose because of several supposedly staphylococcal food poisoning outbreaks in the United States from mushrooms imported from Hong Kong that supposedly were

canned in China. A complete description of the outbreaks and the mushrooms involved was never published. I was told by an authority at FDA that the mushrooms came from Hong Kong and were thought to be blackmarket mushrooms as the lot numbers were incomplete in some cases and different lot numbers were present in the same case. In some outbreaks, the can from which the mushrooms were taken was not available for examination and the mushrooms were not available for testing. Subsequently, a listing of canned mushrooms from several canneries in China that reportedly were positive for enterotoxin appeared in Food Industry Report (Anonymous, 1990). In my opinion, this appears highly improbable as others have examined hundreds of cans of mushrooms from China without ever finding a positive reaction, both with the ELISA ball kit and the RPLA method. Even those who used the TECRA kit, recommended by FDA, did not find any positive reactions. The major possibility of entertoxin being present in canned foods is through cans that are leakers, and I was informed that leakers were found occasionally. It is difficult to understand how enterotoxin could be present in the mushrooms initially or, even if they were, how they could survive the processing. There was a report that, if staphylococci were present in the mushrooms at harvest, these organisms could grow and produce enterotoxin during transportation in sealed plastic bags (Hardt-English *et al.*, 1990). Others were unable to confirm this (Wu, personal communication; Brunner & Wong, 1992). For this to take place, the mushrooms would have to be in transportation for several hours. There is no evidence that staphylococci will grow on raw mushrooms, much less produce enterotoxin. Even if enterotoxin was present on the mushrooms at the time of processing, certainly most of it would be removed during the washing and preparing the mushrooms for retorting. It has been shown that enterotoxin does not survive retorting (Denny, personal communication). It is still a mystery to me how FDA could find so many cans to contain enterotoxin.

This raises a question about the methods used by FDA for enterotoxin detection and the ones they asked the Chinese to use in testing their mushrooms. It was impossible for them to use the "Official Method" because they could do no better than 500 ng of SE with the microslide which is the best many users can achieve. They did purchase 100 of the TECRA kits to be used in China, but this method is not a proven method. They and others obtained false positive reactions with the method. Why FDA recommended this method before it had been adequately tested by other investigators, I do not know. Even FDA was unable to identify blind samples containing enterotoxin using this method (Park, personal communication).

6. INTERNATIONAL COLLABORATION FOR DEVELOPMENT

The commercial kits are quite expensive. Hence, the Chinese desired to develop their own kits for use in China. I was asked to give them an assist in the development of an ELISA kit. I aided them in the purification of the enterotoxins, but they prepared their own enterotoxin antibodies and ELISA kit. I helped them in testing the kit against the ELISA ball kit and the RPLA kit. Their kit was equal in sensitivity to the ELISA ball kit and somewhat more sensitive than the RPLA kit. I congratulate them on their success as I started working with them in October 1990 and helped them test the kit in April 1992. In my opinion it is more reliable that either of the methods required by FDA.

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