RAPID TEST FOR THE DETECTION OF STAPHYLOCOCCAL THERMONUCLEASE IN FERMENTED SAUSAGES

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INTRODUCTION

The nature of the staphylococci, their enterotoxins, and their significance as a major cause of foodborne intoxications have been well documented (15-18). In the USA, there have been at least six incidents of staphylococcal food poisoning traced to fermented sausages (26). It is likely that staphylococcal food poisoning from fermented products occurs sporadically throughout the world as a consequence of starter culture failure or inadequate processing procedures combined with a lack of scientifically controlled culture propogation. Fermented sausage may be prepared in massive volumes and modern technology sometimes utilize elevated processing temperatures that permit the early growth of staphylococci particularly during the green room or initial fermentation process. Processors produce large amounts of product that could on ocassion contain staphylococcal enterotoxin. Accordingly, it is important for the processor to exercise analytical controls to protect himself against financial losses and to protect the public from adulterated product. Viable staphylococcal counts are used by some firms but these can be very misleading.

Our experience has shown that the staphylococcal levels of fermented sausages are extremely variable. We have repeatedly observed wide fluctuations between lots, from stick to stick and even from different locations within an indicidual stick. There are several reasons for these wide fluctuations; first, the sausage meat itself is not homogenous and secondly, the acid by products of the growth of starter cultures are deleterious to the viability of the staphylococci. In general, viability decreases progressively during the drying or ripening of the sausages (25). Consequently, the most inexpensive control procedure for the staphylococci lacks reliability. Control through the detection of staphylococcal enterotoxins is completely unrealistic. These tests require a level of scientific capability that does not exist within the fermented foods industry and the cost is prohibitive particularly in view of the fact that the presence of staphylococcal enterotoxins in the product of any one producer is very unlikely. In recent years, researchers have developed the thermostable deoxyribonuclease (TNase) test which appears to be a reliable method for detecting levels of staphylococcal growth which may have been sufficient for enterotoxin formation (24). Our laboratories have been using this test and have found it to be extremely beneficial. However, because of the extraction step associated with the test, it is not adaptable to high volume testing procedures necessary for industry control purposes or regulatory investigations of large numbers of potentially involved samples. The purpose of this investigation was to attempt to simplify the TNase test as used for fermented sausage.

In 1956, Cunningham et. al. (3) discovered in the culture medium of Staphylococcus aureus (var. pyogenes) a thermonuclease which requires calcium ions for its activity, has a pH optimum of 8.6, and is precipitated from culture fluid with ammonium sulfate. The enzyme is a globular protein consisting of a single polypeptide chain containing 149 amino acids; MW = 16,807 (13). Erickson and Deibel (4) found that thermonuclease production is inhibited by anaerobic incubation and stimulated by aeration and that the enzyme has a D value (90% inactivation time) of 16.6 minutes at 130° C. Some strains of S. epidermidis and Micrococcus sp. produce nucleases. However, thermal stability is unique to the nucleases of S. aureus (11). A remarkable tolerance to prolonged heating, prolonged storage, and bacterial proliferation is exhibited by staphylococcal thermonuclease in foods and broth (13).

Tatini et. al. (24,25), working with several different S. aureus strains and a variety of foods, noted a close relationship between the production of the most commonly occurring enterotoxins in food poisoning outbreaks and thermonuclease production. They reported that thermonuclease is produced by almost all strains of S. aureus, including nearly all (98.3%) enterotoxigenic strains (24). Among 250 enterotoxin - producing strains tested by Lachica et. al. (14), 93% produced coagulase and 95% produced thermonuclease. Niskanen and Koiranen (20) studied 276 S. aureus strains from routine sampling, food poisoning outbreaks, and mastitic milk. All the strains produced thermonuclease and 51% produced enterotoxin. Growth of S. aureus to a level of 10^5 to 10^6 cells per gram of food is necessary before thermonuclease can be detected, and enterotoxins are usually produced in detectable amounts only after the count of staphylococci exceeds 10^6 cells per gram (19).

Many different methods have been developed for detecting thermonuclease in food products. Chesbro and Auborn (2) extracted thermonuclease from foodstuffs with ammonium sulfate followed by precipitation of the enzyme with trichloroacetic acid and spectrophotometric measurement of thermonuclease activity. Jarvis and Lawrence (5) placed a small volume of the enzyme preparation in a well bored in a thin layer of deoxyribonucleic acid agar on a microscope slide. After incubation at 37° C for up to 20 h, the slide was dipped in HCl and the clear zones indicating thermonuclease activity were measured. Thermonuclease extraction procedures have been developed by Koupal and Deibel (7) and Tatini et. al. (24, 25). Park et. al. (21, 22) found that addition of non-fat dry milk to food samples and subsequent precipitation at pH 3.8 enhanced the recovery of thermonuclease from most of 37 foods tested. Lachica et. al. (12) developed a metachromatic agar - diffusion (MAD) microslide technique which was shown to detect nanogram quantities of staphylococcal thermonuclease in various foods without prior extraction, purification, or concentration. Three microliters of liquid food samples or 5 mg of solid food were placed in wells or on the surface, respectively, of slides coated with Toluidine Blue DNA agar. A positive test was indicated by a bright pink halo around the wells or food particles after incubation for three h at 37° C. Among the

thermonuclease assay media used by various researchers are acridine orange-deoxyribonucleate agar (8), methyl green-DNase test agar (23), Toluidine Blue DNA agar used in the metachromatic agar - diffusion methods developed by Lachica et. al. (9, 10, 12), and the agar medium for thermonuclease assay described by Koupal and Deibel (7). Kamman and Tatini (6) reported that use of 50° C and pH 10.0 (instead of 37° C and pH 9.0) in a DNA-agar diffusion system resulted in a twofold reduction in thermonuclease assay time.

When extensive staphylococcal growth occurs in fermented sausage, it is always concentrated in the outermost part of the sausage where oxygen tension is highest (1). Therefore, it is important that sampling procedures involve only the outer 1/8 inch ring of fermented sausages (1, 26). Tatini et. al. (25) found that thermonuclease was detectable in samples of Genoa sausage showing detectable amounts of enterotoxin A. However, enterotoxin was not detected in all samples that showed detectable thermonuclease.

This paper describes a modified TNase test which utilizes casing discs removed from the sausages heated and placed directly upon substrate media without time consuming laboratory procedures. The method is inexpensive, reliable and is nondestructive to the valuable sausages. This rapid test appears to be suitable for economical quality control utilization by sausage manufacturers or private laboratories servicing the smaller manufacturers.

MATERIALS AND METHODS

Media. Toluidine Blue DNA (TB-DNA) agar was prepared as described by Lachica et. al. (9) and Koupal-Deibel DNA (KD-DNA) agar was prepared as described by Koupal and Deibel (7). Each 15 x 100 mm petri dish contained 15 ml of media. Both media were used in all the thermonuclease assay tests. All plates of media were air dried in a laminar flow hood prior to use. All thermonuclease assay plates were incubated at 37° C for 24 h.

Fermented sausages. Six different lots of commercially produced salami naturally contaminated with S. aureus were used in these experiments. Lots A and B were Genoa salami with synthetic fibrous casings; Lots C and D, Hard salami with synthetic fibrous casings; Lot E, Hard salami with collagen casings; and Lot F, Genoa salami with natural casings. A consumer sample of Hard salami (one-third stick) which had caused food poisoning and which was positive for staphylococcal enterotoxin A by radioimmunoassay procedures (R. Mageau, personal communication) was also tested for thermonuclease. All salamis were stored in a freezer prior to testing.

Thermonuclease casing tests. Twenty-four half-inch diameter casing discs were removed from each salami stick. Due to the random location of staphylococcal growth on the surface of fermented sausages (26), care was taken to obtain representative casing discs from the entire stick of salami. The half stick of salami was placed upright in a clean aluminum pie pan during sampling. The disc area was scored with a clean stainless steel cork borer (1/2 inch ID x 4 inches long) and subsequently removed with forceps.

The casing discs were steamed prior to thermonuclease assay. Eight of the casing discs from each salami stick were placed (meat-side-up) on the inside of the lid half of each of two sterile glass petri dishes. The bottom half of the petri dish was then placed (bottom down) inside the lid to press the core samples flat and to hold them in place. The petri dish containing the casing discs was then steamed in an autoclave at 100° C isothermal or over a boiling water bath for 15 min. An aluminum foil cap was placed over the petri dishes during steaming to catch moisture condensation. After steaming, four casing discs were placed (meat-side-down) on the surface of each of the two thermonuclease assay media. A clean glass weight (1/2 inch diameter x 5/16 inch high; cut from a 1/2 inch diameter glass rod) was placed on each casing disc to assure close contact of the casing with the agar surface during incubation. After incubation the glass weights and casing discs were removed, and the plates were examined for thermonuclease activity. For comparison, some casing discs were not steamed.

Thermonuclease extraction and concentration assays. A 20-g sample of meat from the outer eighth-inch shell of each salami stick was tested for thermonuclease according to a modification of the extraction and assay procedure described by Tatini et. al. (24). The extraction procedure was modified by the addition of 10 g $(NH_4)_2 SO_4$ and 2 ml Triton X-100 to the sample prior to blending. Both TB-DNA and KD-DNA agars were used as assay media. A thermonuclease positive boiled Trypticase Soy broth (Baltimore Biological Laboratory, Cockeysville, MD) culture of S. aureus was used as a positive control in each experiment.

Sensitivity of the tests with thermonuclease standards. Known concentrations of staphylococcal thermonuclease (Sigma N 3755) dissolved in Tris buffer (pH 9.0) were placed on half-inch diameter casing discs from a thermonuclease negative lot of Genoa salami. The viable coagulase positive staphylococci counts in the salami at the end of fermentation had ranged from 1150 to 15,000 per g. The enzyme concentration per casing disc was 21.2, 10.6, 5.3, 2.65 or 0 nanograms. The casing discs were held with sterile forceps under a cool air hair dryer as 10 ul of the enzyme solution was dispensed onto the casing discs with a 20-ul pipette. Duplicate casing discs were then placed on each of the two thermonuclease assay media. After incubation the zone diameters of thermonuclease activity were recorded.

Nine enzyme concentrations ranging from 68 to 0.265 ug/ml in two-fold dilutions and a Tris buffer control were added to wells (10 ul per well) in TB-DNA and KD-DNA agars to compare the sensitivity response of these two agars.

RESULTS AND DISCUSSION

In connection with a recent outbreak of staphylococcal food poisoning associated with fermented sausages (27), our laboratory examined a large number of salami sticks for thermonuclease by the extraction procedure. Our experience from this work indicated that sample preparation and extraction steps for the thermonuclease assay were quite laborious. As a result, efforts were made to develop a simple, rapid thermonuclease test for fermented sausages which would be as sensitive and reliable as the extraction procedure. Fortunately, this outbreak made available a large number of salami samples naturally contaminated with S. aureus, thermonuclease, and enterotoxin for this developmental work.

It has been shown that staphylococcal growth in fermented sausages occurs in the outermost part of the sausage where oxygen tension is highest (1). In addition, it is probable that thermonuclease is concentrated by moisture evaporation at the meat-casing interface during the drying of fermented sausages. We therefore tested samples of salami casings (without any meat) directly on the surface of the thermonuclease assay plates. A salami sample which was positive for enterotoxin A (R. Mageau, personal communication) and thermonuclease by the extraction procedure also showed a strong thermonuclease reaction when casing discs from the sample were placed directly on the two thermonuclease assay media. We observed that the zones of TNase activity were not always symmetrical.

The KD-DNA agar proved to be somewhat superior to TB-DNA agar for thermonuclease assay of salami casings. When the casing discs were removed from the surface of the TB-DNA agar after incubation, colorless to light blue zones were found directly under the casings, whereas the pink color indicative of thermonuclease activity was prominent only around the perimeter. In some cases the pink color was very light which made the assay results questionable. Other researchers have reported similar problems with proper color development in the metachromatic agar-diffusion system (7). The thermonuclease reaction was more pronounced on the KD-DNA agar because the zones of clearing after the precipitation of nonhydrolyzed DNA with 1N HCl were found both directly under as well as around the perimeter of the casing discs. The clear zones were very distinct when held against a dark background. The only disadvantage observed with the KD-DNA agar is that once the plates are flooded with 1N HCl, they cannot be reincubated. This problem is not encountered with the TB-DNA agar assay system. Table 1 shows the zone diameters (mm) of thermonuclease activity from fermented sausage casing discs containing known concentrations of staphylococcal thermonuclease. Zone diameters were identical on TB-DNA and KD-DNA agars. Zones of thermonuclease activity were detectable at thermonuclease concentrations of 2.65 ng of enzyme per half-inch diameter casing disc. Again the pink color development on TB-DNA agar was poor (very light pink to almost colorless) at this low concentration level, but the zones were very distinct on KD-DNA agar. Steaming the casings prior to assay caused a slight decrease in zone diameters on both assay media. The results in Table 1 demonstrated that about 2.5 to 5 ng of staphylococcal thermonuclease could be detected on the salami casing discs and that the sensitivity of this method was comparable to the levels reported previously (7, 12, 22).

Six lots of salami with thermonuclease positive and negative sticks were chosen to evaluate the thermonuclease casing tests. The results presented in Table 2 showed that the thermonuclease casing test results were in complete agreement with the results of thermonuclease assay by the extraction procedure. However, about 50 samples could be tested in a day by the thermonuclease casing method compared to only about five samples per day by the extraction procedure. Steaming the casing discs prior to thermonuclease assay was necessary to eliminate false positives in Lots D and E (Table 2).

Although the thermonuclease casing test is not a quantitative assay, it is a simple and rapid test that can be readily incorporated into quality control programs. There would be little need in a food industry quality control program for a quantitative thermonuclease test. Different strains of S. aureus produce different levels of thermonuclease (4), so there can be no direct correlation between S. aureus numbers and the amount of thermonuclease detected. This test is intended as a screening test for qualitative presence of thermonuclease which dictates the need for further testing for presence of staphylococcal enterotoxins.

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TABLE 1. Zone diameters (mm) of thermonuclease activity from fermented sausage casing discs containing known concentrations of staphylococcal thermonuclease

Enzyme concentration (ng)	Not steamed		Steamed in autoclave		Steamed over boiling water bath	
	TB-DNA[a]	KD-DNA[b]	TB-DNA	KD-DNA	TB-DNA	KD-DNA
21.2	23[c]	23	21	21	20	20
10.6	21	21	20	20	19	19
5.3	19	19	18	18	17	17
2.65	17	17	16	16	15	15
0	0	0	0	0	0	0

[a]TB-DNA = Toluidine Blue DNA agar
[b]KD-DNA = Koupal-Deibel DNA agar
[c]Diameter of casing disc = 12.7 mm

TABLE 2. Comparison of thermonuclease casing tests with the thermonuclease extraction and assay method on six lots of commercially produced fermented sausage

Salami Sample Number	Thermonuclease extraction method		Casing test (not steamed)		Casing test (steamed in autoclave)		Casing test (steamed over boiling water bath)	
	TB-DNA[a]	KD-DNA[b]	TB-DNA	KD-DNA	TB-DNA	KD-DNA	TB-DNA	KD-DNA
LOT A								
A-1	+[c]	+	++	++	++	++	++	++
A-2	+	+	±	±	±	±	±	±
A-3	+	+	±	±	±	±	±	±
A-4	+	+	+	+	+	+	+	+
A-5	+	+	+	+	+	+	±	+
LOT B								
B-1	+	+	±	+	+	+	±	+
B-2	+	+	±	+	±	+	±	+
B-3	+	+	+	+	±	+	±	+
B-4	+	+	+	+	±	+	±	+
B-5	+	+	±	+	±	+	±	+
LOT C								
C-1	+	+	+	+	+	+	+	+
C-2	+	+	±	±	±	±	±	±
C-3	+	+	±	±	±	±	±	++
C-4	+	+	±	+	±	±	±	+
C-5	+	+	±	+	±	±	±	+
LOT D								
D-1	-	-	-	-	-	-	-	-
D-2	-	-	-	-	-	-	-	-
D-3	-	-	-	-	-	-	-	-
D-4	-	-	-	-	-	-	-	-
D-5	-	-	-	±	-	-	-	-
LOT E								
E-1	-	-	-	-	-	-	-	-
E-2	-	-	±	±	-	-	-	-
E-3	-	-	±	±	-	-	-	-
E-4	-	-	-	±	-	-	-	-
E-5	-	-	±	±	-	-	-	-
LOT F								
F-1	-	-	-	-	-	-	-	-
F-2	-	-	-	-	-	-	-	-
F-3	-	-	-	-	-	-	-	-
F-4	-	-	-	-	-	-	-	-
F-5	-	-	-	-	-	-	-	-

[a]TB-DNA = Toluidine Blue DNA agar
[b]KD-DNA = Koupal-Deibel DNA agar
[c]++ = strongly positive (>20 mm zone diameter); + = moderately positive (15 to 20 mm zone diameter)

± = weakly positive (<15 mm zone diameter), - = negative (no zone)