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Expanding the Use of Validated Rapid Microbiological Methods to New Food Matrices

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What I'd like to talk about today is some of the work that we're doing at NMSU. We're looking at rapid methods for pathogen detection. A lot of rapid microbiological methods were developed for use in meat or dairy products or some other high-end items. We're examining their performance in different food matrices such as vegetables like cilantro or alfalfa sprouts. So we're taking methods that were validated for use in one or more food products and trying them with other foods. As Bob mentioned, our lab is in Las Cruces which is very pretty close to the Mexican border. This gives us a lot of opportunities for examining foods coming across the border.

Currently we have a project with students in our lab looking for *Staphylococcus aureus* in raw milk cheeses that are made across the border. Lots of cheese is being brought into the country by people for personal use and we're trying to examine the impact of that. The Food Safety Lab is part of the Physical Science Laboratory at NMSU and there are six analysts and three students working in the lab. The types of contaminants that can affect food safety are bacteria, viruses, toxins, chemicals and radio nuclear agents. In the main, we work with bacterial contaminants. Air, water, food and food distribution systems are all possible means for delivering these agents.

What is a rapid method? In general, any method that is faster than the traditional methods of detection. We're trying to find new applications for existing methods because a lot of the standard surveillance work is slow and tedious using the cultural methods. If we can speed up some of the methods, this will free up time for people in the laboratories. Of course we are looking for methods that are fast and reliable. These methods come in all sorts of shapes and sizes. Many require that the user buy a lot of equipment and a computer and to go along with them. Others are very simple like these lateral flow devices and so, we're looking at a variety of them.

Now, when it comes to detecting microorganisms with rapid methods, there are a number of potential targets, such as, the physical structure of the organism itself, metabolites that the organism makes, proteins, DNA and RNA. Rapid microbiological methods can be broken down into four broad categories: antibody-based methods, DNA or nuclear acid-based methods, biochemical and enzymatic methods and membrane filtration techniques. We conduct single lab validation studies of rapid methods. Most of the methods that we're examining have already been through extensive validation, but for different foods than what we're looking at. We're trying show that we've got good confidence in the rapid method by comparing it to a reference method. Typically

the reference method we use for comparison is the standard cultural procedure, that is, the gold standard.

There are 2 different types of methods— qualitative and quantitative methods. With the qualitative methods we are looking for a plus/minus answer. Is it there or not? In other cases we're trying to measure how much of the analyte is present in a sample. These are quantitative methods. We usually enumerate microorganisms. Methods that are listed as appropriate for "all foods" may not have been validated against certain foods. And, methods are listed as for specific foods have only been validated for those foods. We have evaluated several quantitative methods that had already been validated for use in certain foods. The companies that developed the tests have already done a lot of work to make sure that the test was able to detect very wide range of organisms within the species that we were looking for. That the test be inclusive. Also, to ensure that competitive microorganisms are excluded and not interfere with the test. What we try to do is find new food types for these tests to be used in.

To set up an experiment to validate a quantitative method, we inoculate the food at three levels and one portion we don't seed at all and that will be our uninoculated control. At the low level we seed the organism at a level that is somewhere at the bottom end of the detection limit and then we'll have medium and high levels which are inoculated at levels that are one and two log units higher. For each test we spike five samples at each of these levels test the samples by both the reference method and the alternative method (which is our rapid test).

One of the first validations we did in our laboratory was a most probable number technique. We examined the ColiComplete test for enumeration of *E. coli* and total coliforms. Basically, we inoculated the food samples and diluted them down to a ten to the minus five dilution. For the conventional test we took one ml portions of each dilution and inoculated them into 3 tubes of LST broth. For the ColiComplete test we put the dilutions into LST broth containing the ColiComplete substrate supporting discs. The ColiComplete discs contain a lactose surrogate and in the tubes containing coliforms you see nice blue derivatives that precipitate in the tube. There is also a fluorogenic substrate on the discs that detects *E. coli* by its glucuronidase activity. With this test we get fast, confirmed, *E. coli* results in 28 to 30 h and that's a lot faster than the typical MPN procedure for *E. coli* that's in use. When you look at the collaborative study that was done on the method, it was conducted with dry eggs, frozen broccoli, nut meals, raw milk, ground beef and pork sausage. So, we weren't sure if there was any good data for other products.

We tested the method with five or six different products. The first one was cheese. We tried mold ripened cheeses because we knew that that was a pretty nasty food matrix. These cheeses contain molds, all the enzymes they produce and other substances produced during cheese ripening that might interfere with the test. We spiked the samples, made dilutions and performed each of the methods on the diluted samples. The BAM method takes a lot longer than the ColiComplete method to do. For *E. coli*, we have to grow our LST tubes, transfer them to EC broth, then onto EMB agar to get bacterial isolates. We have to confirm that the bacterial isolates are in fact *E. coli*. The whole procedure takes about a week. For the ColiComplete method we examine the LST tubes under UV light after 28 h incubation and if they fluoresce, then there is *E. coli* in the sample. For coliforms we wait until 48 h and look for the blue precipitate for a confirmed coliform test. But with the conventional coliform test, we have to subculture the LST tubes into Brilliant Green Lactose Bile Broth and look for growth and gas production. The ColiComplete method is easier to perform.

When we tried the method with gorgonzola cheese we found that we had pretty good agreement between the two tests. We compared the results of the two methods using paired t-tests and calculated probabilities that the two methods were equally effective at enumerating *E. coli* in the spiked samples. No statistical differences were found between the two methods for enumerating *E. coli* in gorgonzola cheese. The same thing happened when we were enumerating *E. coli* in parsley and so we're kind of happy the way that worked out. I didn't show the results for the coliforms, however the test worked well on the gorgonzola cheese and all of the other cheeses we tried. The only coliforms in the cheeses were those that we added. Whereas parsley and some other raw vegetable products had high background levels of coliforms and for those the test didn't work as well.

Another quantitative test that we evaluated was the Petrifilm *E. coli*/coliform count plate. Although it is a familiar test that has widespread use we weren't certain if it would work with certain foods. These plates are very convenient to use. You have to make a dilution of your sample and inoculate that onto the Petrifilm plate. The plates contain all of the violet red bile agar ingredients except agar and instead contains a gelling agent. *E. coli* turns blue on these plates because they contain a glucuronidase indicator. Gas production by coliforms is detected by the presence of little gas bubbles which are trapped in the plate. Now, these plates have been validated for use on all kinds of foods. The foods that were involved in the collaborative study were ground turkey, raw mushrooms, beef with gravy, cheese, wheat flour and nut meal. We looked their performance with a number of hard cheeses and produce samples. We compared the *E. coli*/coliform count plates with the VRBA MUG direct plating procedure. For both parmesan cheese and parsley, the two methods compared well with one another for *E. coli* enumerations. When it came to enumerating coliforms in parmesan cheese, we had a pretty good match. For parsley the two methods were found to be statistically different from one another with higher counts consistently being found with the VRBA BAM procedure. However, even though the results from the two methods were found to be different, the plate counts were still within one log cycle. So it wasn't such a great technique for coliform determinations in raw parsley.

The other broad category of rapid tests that we perform are qualitative methods which give a plus/minus answer—is it there or not? Like with the qualitative tests that have been approved for use in foods, the methods have many strengths and have already been shown to be valid for certain applications. We look to see if they work in other foods than those that they have already been shown to work with. The way that we run validation tests for these is that we inoculate 20 samples at a low inoculum level, 20 samples at a high inoculum level and we also test five uninoculated control samples. At the low level we seed the organisms at 1 to 5 CFU/g and at the high level we use 10 to 50 cfu/g of food.

The way that we determine our inoculum levels is by the most probable number technique. We inoculate at fairly low levels, 1 CFU per 25 grams of food which is 0.04 CFU's per gram so we can't enumerate our inoculum in our samples by plating. We do MPN's on our inoculated samples to enumerate our added organisms. To do so we use large samples for our MPN's. We have to start with 100g, 10g and 1 g portions for MPN determinations and we do those in triplicate. So we have to use a lot of sample and a lot of broth to get our MPN values.

One of the tests we tried in our lab was a VIDAS test for *Salmonella* detection. In this method an immunoconcentration method is combined with an enzyme linked immunofluorescent assay. To do this assay we grow up an overnight enrichment of our sample, put a portion of it into the VIDAS ICS strip and place the strip into the VIDAS machine. Antibodies directed against *Salmonella* spp. are used to collect the organism from the enrichment and deposit them into a well on the strip. We take a sample of the concentrated sample and place it into another broth. After three or four hours of post enrichment we take a sample and boil it and use it to run a standard VIDAS Salmonella immunoassay which has been shown to work quite well. If you look at the information about this combination of tests you find that it's kind of complicated. The foods used in the collaborative study used to validate the test were milk chocolate, non fat dry milk, black pepper, dried whole egg, soy flour and raw turkey. Yet the test can be used on the very specific foods listed here. We wanted to try and use it for some different food items, particularly since it's a rather quick test. You can get results in about 26 hours. For screening produce items coming across the border everyone wants more rapid Salmonella methods.

So we inoculated some produce items with Salmonella and compared the rapid method with the cultural procedure for detecting the inoculated organisms. For many produce samples we use a rinse technique for preparing our samples. What we do is add sterile buffer to our inoculated samples, mix them and use a portion of the liquid to test by the two methods. The VIDAS assay is considerably faster than the standard cultural method for Salmonella detection. For the cultural method, the first step is a non selective enrichment of the sample, followed by selective enrichment and then plating onto selective agar plates. After those are grown up it is necessary to screen the isolates and go through confirmation of the isolates. We usually use API 20E test strips and serology to confirm our isolates. The whole procedure takes about a week for positive samples. For the VIDAS test, we get results in a little over a day.

We tried the VIDAS immunoconcentration and fluorescence assay on a number of foods. I'm just showing you two here. We tried it on mung bean sprouts. Mung bean sprouts contaminated with Salmonella have been involved in some foodborne disease outbreaks. We wanted a fast way of detecting Salmonella in them. It didn't turn out to be this method because of our 20 spiked samples that had been inoculated at 2.3 MPNs per 25 grams; 15 were positive by the BAM procedure, however none of them came up positive by this method. So it didn't seem to work very well. We did a chi square analysis on the data and found a χ^2 value of 15 which is not very good. Anything greater than 3.84 is considered to be significantly different. We also used the method with inoculated lettuce. At the low inoculum level, 1.1 MPN per 25 grams, 17 of the 20 samples were positive by the BAM and 15 by the VIDAS method. Our chi square value was 0.5, but still 2 of the inoculated samples were being missed by the rapid method. You have to be very careful when using this method for produce samples.

Another Salmonella method we evaluated was the Salmonella UNIQUE assay which is a Salmonella immunoassay on a dipstick. It comes in ready-to-use self-contained modules that have everything in them. What you do is grow up an overnight enrichment culture of your sample and use that for the test. It uses a specific broth with or without the use of additional additives and the manufacturers give

recommendations on how to use them. For instance if you're testing a food containing fat, there's an additive; if you're testing a food that has a high background microflora you have to use elevated incubation temperatures and different additives. So they have tweaked their enrichment step but it's still a single enrichment step.

Following enrichment you put a portion the enriched sample into the first well and then you put one of their little sticks into it. It's a dipstick that's got antibodies against Salmonella on it. After collecting the Salmonella on the dipstick it goes through a couple of wash steps and then is transferred into a broth. After a few hours of incubation the dipsticks are developed and if there is Salmonella in the sample then a nice line shows up on the dipstick which indicates a positive result. We used this test on a number of different food products such as cilantro and strawberries. Using this test we found a couple of samples where the samples came up positive on the test device but we were unable to isolate the organism from the enrichment broth. We only put the confirmed results here. At the low inoculum level we found 15 of 20 inoculated samples positive by the UNIQUE test but 17 were positive by the BAM. For strawberries, 16 by this test but 17 by the BAM. Because the test is made with polyclonal antibodies against Salmonella there is some cross reactivity. It's a great test for screening but you have to confirm the results.

In addition to the antibody based techniques, we've also used some DNA-based methods for pathogen detection. One that we tried was the BAX method. The BAX Salmonella test is a PCR procedure. All of the PCR reagents are packaged inside the PCR reaction tubes so it is convenient to use. For this method we follow a standard enrichment of the sample, like is done in the BAM, and then boil up a portion of the enrichment broth to lyse the cells and release the DNA. We hydrate the PCR tablets that come with the test with the lysate and then process the samples in the automated unit. This is a real time PCR procedure that uses SYBR green for detection so we have to do a melting point analysis at the end of the run to show that the amplified DNA is what we wanted. The nice thing with this test is that it gives a positive or negative result so there's no interpretation necessary; that's kind of nice. It worked very well when we did use it with inoculated milk and strawberries. We had identical results by the BAM and the BAX so we were pretty pleased with that.

We also do method development work in our lab. We're working with microbiologists from the Gulf Coast Seafood Laboratory on a method for detection of toxigenic *Vibrio cholerae* in foods. They developed the method for use in seafood and we are trying it with some different foods as well. The method involves a short enrichment of the sample in alkaline peptone water and real time PCR on the boiled enrichment culture. The method uses oligonucleotide primers and a fluorogenic probe that targets a region of the cholera toxin gene. The method also includes the use of an internal control which is used to show if the PCR reaction proceeded as expected.

When we tried the method with potato salad we prepared sample enrichments using a 1 to 10 dilution ratio for enrichment. We took 25 grams of our inoculated sample and enriched it in 225 ml of alkaline peptone water. We saw that all those came up negative by PCR. We weren't able to detect the toxin in the enrichments, but the internal control result was increased. Normally it takes about 20 to 22 cycles to get a positive result for it, but we weren't seeing that in this case. So this shows a case of PCR inhibition. We were able to detect the toxin gene and get reliable results with the internal control when we grew our enrichment at a 1 to 100 ratio instead. So we were able to get rid of the inhibitory activity when we moved to using the diluted sample enrichment.

That's about all I've got to say except that there's a lot of rapid methods that have been developed and for these methods to be reliable in certain foods they have to be tested in those foods. Certain food matrices contain all kinds of stuff in them that can interfere with detection like background microorganisms and inhibitors, especially for PCR. For some tests some sort of clean up step might be needed. What we try to do is make the method as simple as possible to avoid a lot of extra work to get reliable rapid test results.

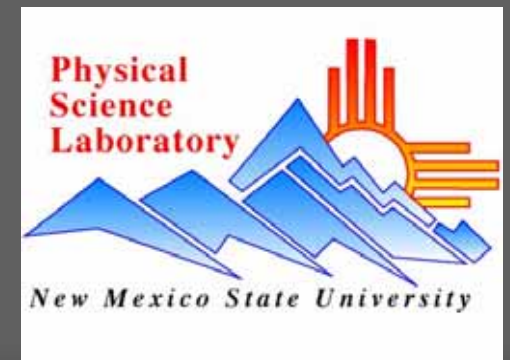
Many rapid methods require an enrichment step. Enrichments increase the time required for the test but are useful because they can help reduce the effect of inhibitors and can increase your chances of recovering injured cells from certain food samples. Bacterial cells that are injured or stressed during food processing might have a chance to recover during enrichment culturing be better detected.

I guess the take-home message is that rapid microbiological methods that are intended for use in all foods should be checked in foods one-at-a-time to make sure that they work.

Expanding the use of validated rapid microbiological methods to new food matrices



Willis Fedio
Laboratory Director
Food Safety Laboratory



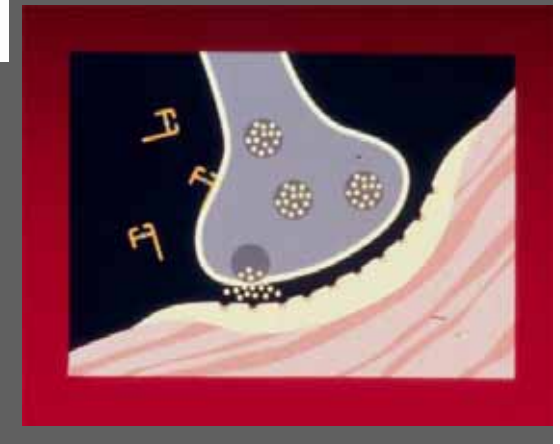
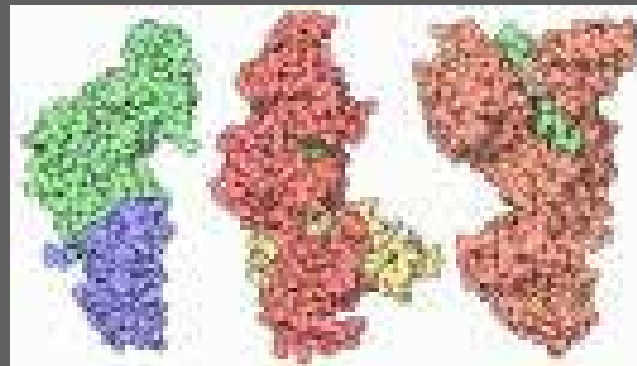
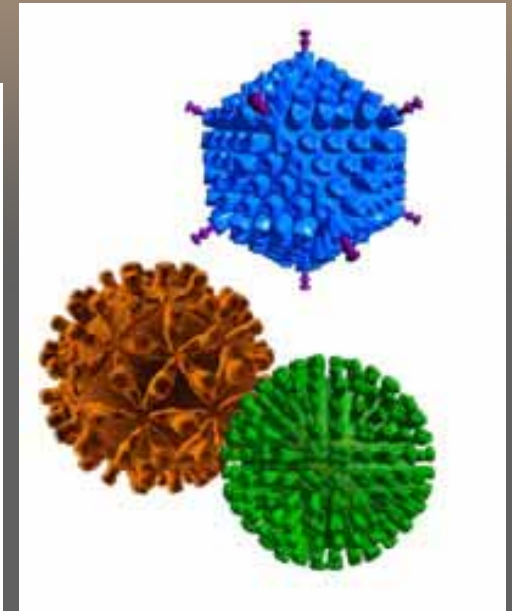
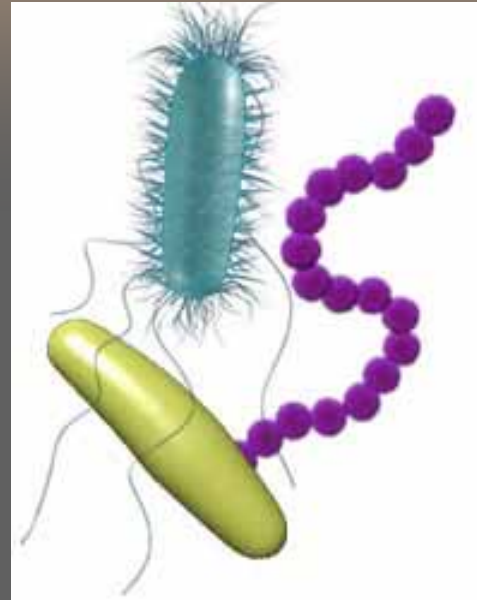


PSL's Food Safety Laboratory



Types of agents

- Bacteria
- Viruses
- Toxins
- Chemicals
- Radionuclear agents



Delivery

- Air
- Water
- Food
- Distribution systems



What is a rapid method?

- Generally, any method faster than traditional methods of detection
- Important properties when evaluating any method:

speed

sensitivity

specificity

capacity

automation

simplicity

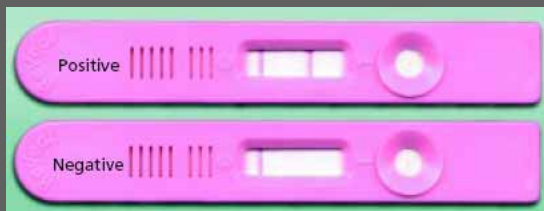
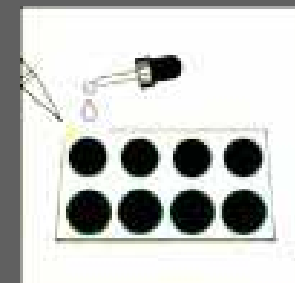
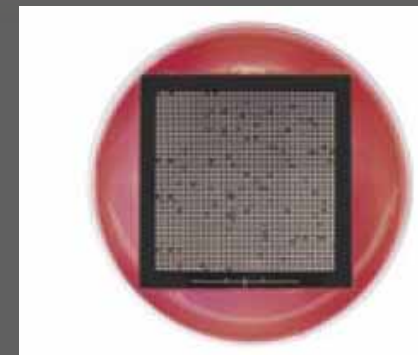
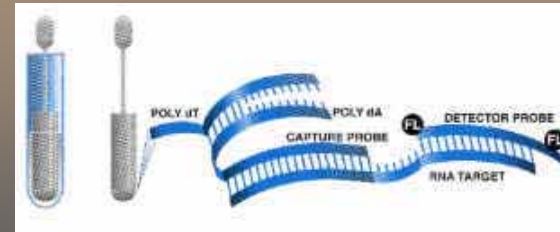
safety

cost

reproducibility

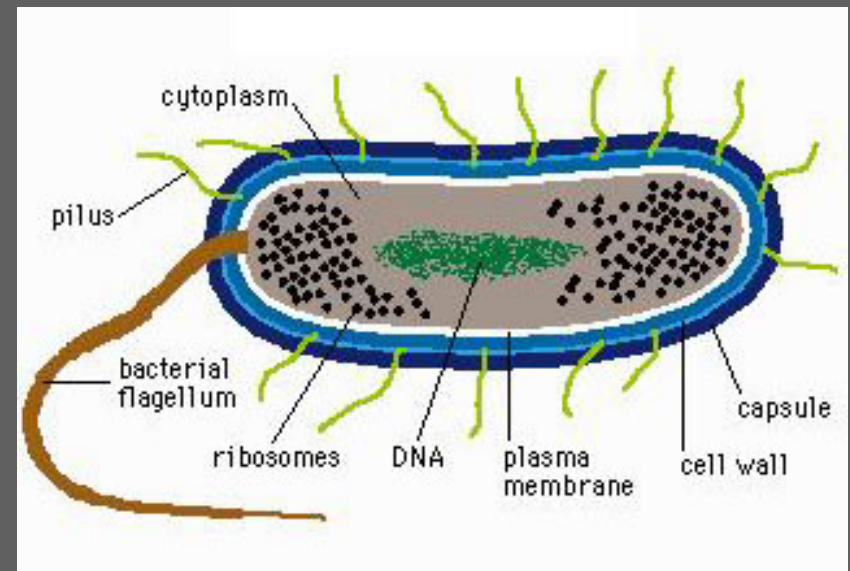
quantitative vs qualitative

viable vs non-viable



Rapid detection targets

- Physical structure
- Metabolites
- Proteins
- DNA
- RNA



Rapid methods

- Antibody-based techniques
- Nucleic acid-based techniques
- Biochemical and enzymatic methods
- Membrane filtration

Validation of alternative methods

- Demonstration that adequate confidence is provided when the results obtained by the alternative method are comparable to those obtained using the reference method using the statistical criteria contained in the approved validation protocol (Feldsine *et al.*, 2002)

Qualitative method

- Method of analysis whose response is either the presence or absence of the analyte (microorganism or associated byproducts) detected either directly or indirectly in a certain amount of sample

Quantitative method

- Method of analysis whose response is the amount of the analyte measured either directly (e.g. enumeration in a mass or volume) or indirectly (e.g. color, absorbance, impedance, etc.) in a certain amount of sample

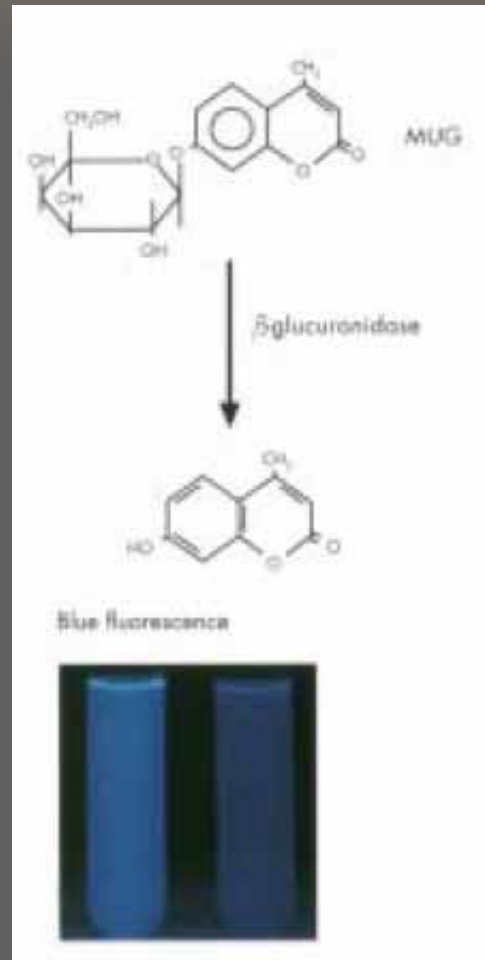
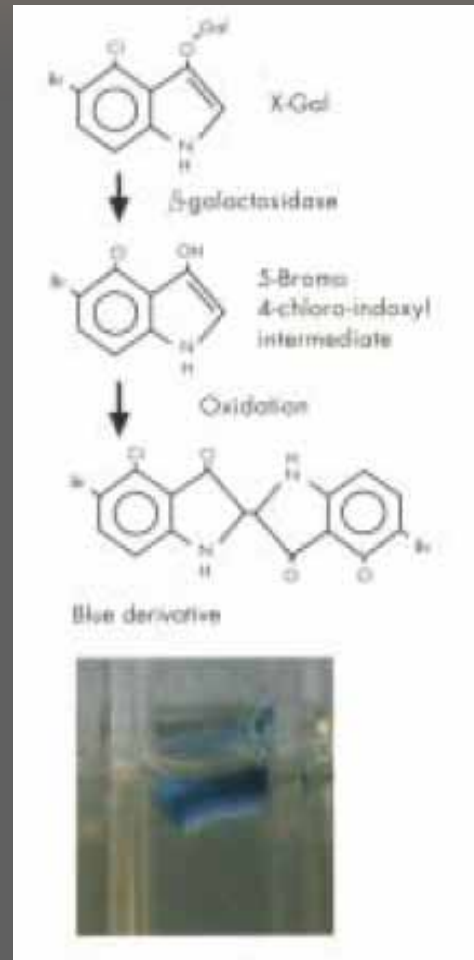
- Methods for “all foods” may have not been validated against certain foods.
- Methods for specific foods have only been validated for those foods.

Quantitative methods

- Applicability
 - Target analyte--inclusivity/exclusivity
 - Food type
- Levels of inoculum
 - Uninoculated
 - Low (at limit of detection)
 - Medium (one log unit higher)
 - High (two log units higher)
- For each, test 5 samples by the reference method and 5 samples by the alternative method



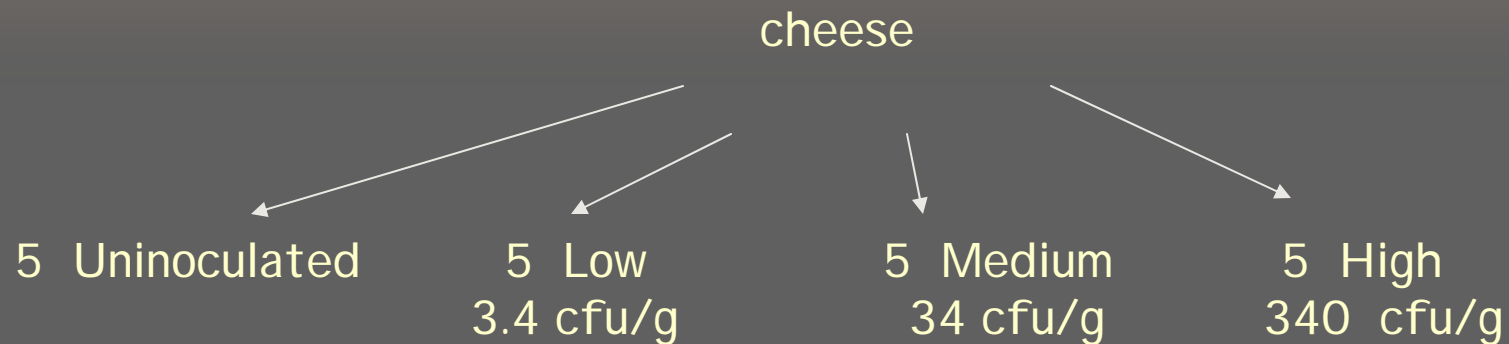
ColiComplete® — *E. coli* and total coliforms



- Substrate supporting disc used with Lauryl Sulfate Tryptose broth tubes used in MPN enumeration
- Chromogenic assay for galactopyranosidase (X-gal)
- Fluorogenic assay for glucoronidase (MUG)
- Fast confirmed *E. coli* results in 28 h

Dry egg, frozen broccoli, nut meats, raw liquid milk, ground beef, pork sausage

FDA-BAM method and ColiComplete® method for enumeration of *E. coli* and total coliforms in cheese artificially contaminated with *E. coli* 25922



10^{-1} to 10^{-5} Dilutions

FDA-BAM Method
(LST, EC, IMViC, EMB, BGLB)

ColiComplete® Method
(LST + SSD)

24h, 48 h blue (X-Gal)
30h uv (MUG)

Enumeration of *E. coli* by ColiComplete[®] and FDA-BAM

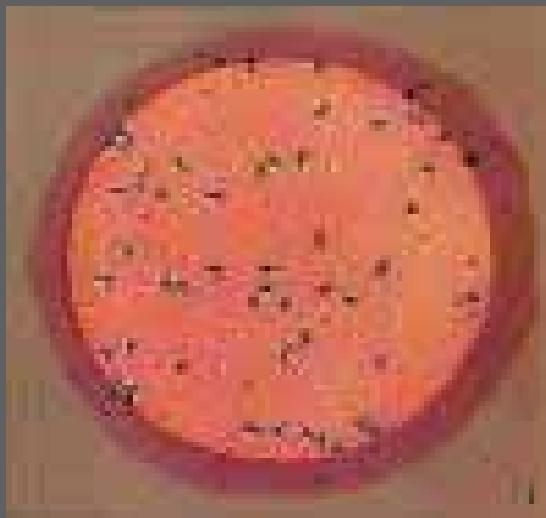


Food	Inoculum	<i>E. coli</i>		Probability
		BAM	ColiComplete [®]	
		Log MPN/g ± SD		
Gorgonzola	Uninoculated	0.48±0	0.48±0	
	Low	0.62±0.19	0.70±0.37	0.73
	Medium	1.86±0.37	1.59±0.25	0.09
	High	2.88±0.40	2.76±0.03	0.68
Parsley	Uninoculated	0.50±0.12	0.48±0.02	0.33
	Low	0.80±0.33	0.73±0.33	0.16
	Medium	1.42±0.57	1.60±0.70	0.13
	High	2.70±0.85	2.50±0.75	0.09

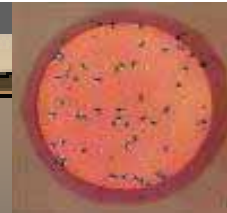
E. coli /coliform plate



- ready-made culture medium system which contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, an indicator of glucuronidase activity, and a tetrazolium indicator
- the top film traps gas produced by the coliforms
- a glucuronidase indicator forms a blue precipitate around *E. coli* colonies are present



Ground turkey, raw mushroom, beef with gravy, cheese, wheat flour, nutmeal



Evaluation of *E. coli*/Coliform Petrifilm Count Plate

Food	Inoculum	<i>E. coli</i>		Probability
		BAM	Petrifilm	
		Log CFU/g ± S D		
Parmesan	Uninoculated	0.99±0	0.99±0	
	Low	1.67±0.16	1.82±0.16	0.13
	Medium	2.64±0.10	2.67±0.09	0.15
	High	3.61±0.11	3.66±0.06	0.21
Parsley	Uninoculated	0.48±0	0.48±0	
	Low	0.44±0.08	0.51±0.65	0.81
	Medium	1.94±0.30	1.99±0.34	0.85
	High	2.57±1.29	3.22±0.15	0.29



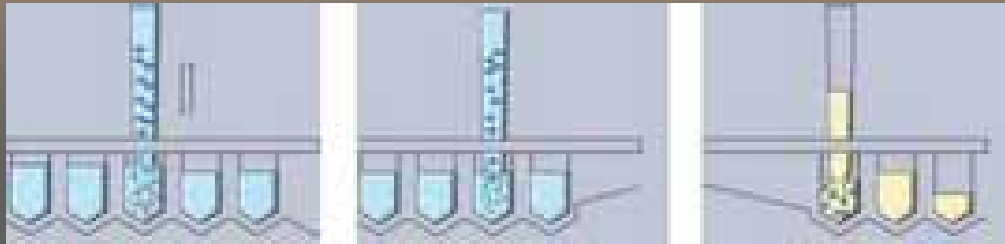
Evaluation of *E. coli*/Coliform Petrifilm Count Plate

Food	Inoculum	Coliforms		Probability
		BAM	Petrifilm	
		Log CFU/g ± SD		
Parmesan	Uninoculated	0.99±0	0.99±0	
	Low	1.67±0.16	1.82±0.16	0.13
	Medium	2.64±0.10	2.67±0.09	0.15
	High	3.61±0.11	3.66±0.06	0.21
Parsley	Uninoculated	3.73 ±0.95	2.82±0.56	0.01*
	Low	3.68±0.17	2.87±0.25	0.01*
	Medium	3.74±0.51	3.12±0.34	0.07
	High	3.61±0.39	3.47±0.35	0.03*

Qualitative methods

- Applicability
 - Target analyte--inclusivity/exclusivity
 - Food type
- Levels of inoculum
 - Uninoculated
 - Low (1-5 cfu/25g)
 - High (10-50 cfu/25g)
- 20 test samples at high and low inoculum levels, 5 test samples for uninoculated

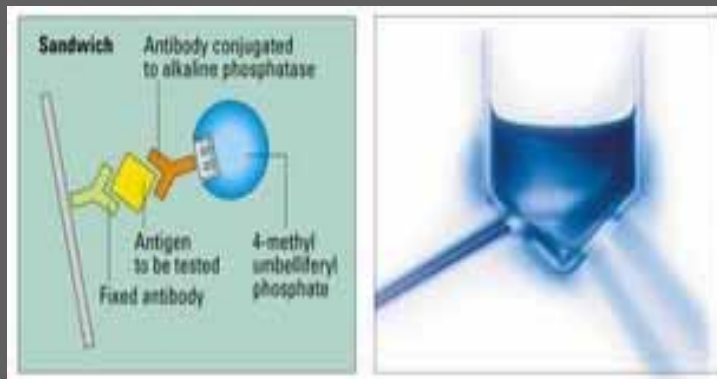
VIDAS Immunoconcentration -- *Salmonella*



Following preenrichment, immunoconcentration of the Salmonella in the sample takes place

Postenrichment, boil sample

Enzyme Linked Fluorescent Immunoassay for detection



Collaborative Study:

Milk chocolate, NFDM, black pepper, dried whole egg, soy flour, raw turkey

Use for:

NFDM, cheese powder, pecans, roast beef, shrimp, fish, peanut butter, pork, casein, cocoa powder, soy flour, dry pasta, egg powder, chocolate, black pepper, gelatin, meat and bone meal, cauliflower, orange juice, turkey, pork sausage

Salmonella VI D A S (I C S / S L M) method for the detection of *Salmonella* spp. in foods artificially contaminated with *Salmonella* Typhimurium

Food
(in sterile bag)

500g

500g

500g

Innoculation with
Salmonella Typhimurium

Control

Low

High

(1-5 cfu/25g)

(10-50 cfu/25g)

Add sterile buffer and mix

Rinse

FDA BAM method

VIDAS method

Evaluation of VIDAS ICS-SLM—*Salmonella*



Food	Inoculum	MPN/25g	Samples	BAM	ICS-SLM	χ^2
Mung	Control	< 0.003	5	0	0	
Bean	Low	2.3	20	15	0	15
Sprouts	High	60	20	20	19	0.05
Lettuce	Control	< 0.003	5	0	0	
	Low	1.1	20	17	15	0.5
	High	23	20	20	20	UD

Salmonella UNIQUE™

- Immunoenrichment™ technology
- One simple enrichment step- mBPW, additives, elevated temperature
- Results in 22 hours
- Built in positive and negative controls
- AOAC approved for all foods
- Collaborative Study: NFDM, black pepper, soy flour, milk chocolate, dried whole egg



- ready-to-use
- self-contained modules

Evaluation of TECRA UNI QUE--*Salmonella*



Food	Inoculum	MPN/25g	Samples	TECRA	BAM	χ^2
Cilantro	Control	<0.003	5	0	0	
	Low	3.8	20	15	17	0.25
	High	7.3	20	20	20	UD
Strawberry	Control	<0.003	5	0	0	
	Low	1.1	20	16	17	0.5
	High	23.3	20	20	20	UD

BAX® - *Salmonella*

- PCR reagent tablets packaged inside the PCR tubes contained in each kit
- After standard enrichment, samples are lysed to break open the cell walls and release DNA
- This lysate is then used to hydrate the PCR tablets
- Processing in the automated unit takes less than 4 hours for a full rack of 96 tests
- Electronic results appear as positive/negative icons on screen
- SYBR Green



AOAC Official Method 2003.09
collaborative study on raw beef,
raw chicken, raw frozen fish,
cheese, frankfurters and
orange juice

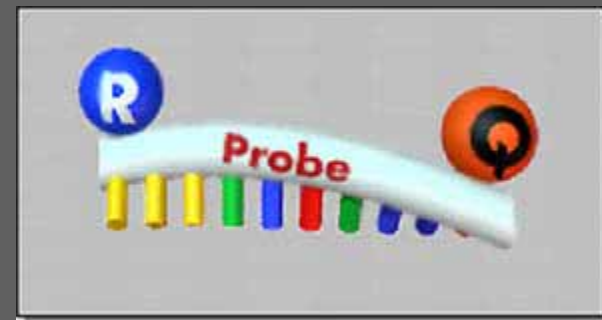
Evaluation of BAX--- *Salmonella*



Food	Inoculum	MPN/25g	Samples	BAM	BAX	χ^2
Milk	Control	< 0.003	5	0	0	
	Low	1.9	20	10	10	UD
	High	5.3	20	20	20	UD
Strawberry	Control	< 0.003	5	0	0	
	Low	1.1	20	17	17	UD
	High	23.3	20	20	20	UD

RT-PCR for detection of toxigenic *Vibrio cholerae*

- Short enrichment of samples in Alkaline Peptone Water followed by RT-PCR using boiled enrichment culture
- Oligonucleotide primers and fluorogenic probe targeting an 84 bp region of the cholera toxin (ctx) gene



Detection of *Vibrio cholerae* C6707 in artificially contaminated potato salad (6h enrichment at 35°C)



Dilution	Sample	Real Time PCR	
		Ct	
		ctx	Internal Control
1:10	A	0	33.64
	B	0	37.46
	C	0	36.11
	D	0	38.29
	E	0	27.37
	F	0	22.97
1:100	A	31.43	21.47
	B	30.49	21.79
	C	31.14	23.61
	D	31.73	20.88
	E	31.55	20.65
	F	30.11	20.45

Conclusions

- Many rapid microbiological methods have been developed
- For these methods to be reliable, extensive testing is necessary
 - inclusivity and exclusivity of the organisms of interest
- Most rapid methods require an enrichment step which increases the time for the test but
 - can dilute the effect of inhibitors
 - allows differentiation of viable from non-viable cells
 - allows for repair of cell stress or injury that may have resulted during food processing
- Rapid microbiological methods approved for use in “all foods” may not perform reliably for certain foods due to:
 - background microorganisms
 - matrix inhibition

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