EVALUATION OF METHODS FOR DETECTION OF COAGULASE POSITIVE *STAPHYLOCOCCUS* AND STAPHYLOCOCCAL TOXIN IN MILK AND CHEESE

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1 EXECUTIVE SUMMARY

This report details the available methods for the detection of coagulase-producing staphylococci and their enterotoxins that could be applied at various stages during cheese production from the view of their possible use by small cheese makers.

For the organism itself there are three broad groups of approaches; conventional cultural, immunocapture and molecular detection. Conventional culture is unlikely to be an appropriate technique because of the range of equipment needed; these methods were designed to be used by fully equipped food testing laboratories. Some simplification of this has occurred with the introduction of a film-based disposable "agar plate" but specialised equipment and training are still required. A similar consideration applies to immunocapture methods such as ELISA. Molecular methods, despite being available in kit form, tend to require the use of expensive equipment which may cost in the order of $100,000.

Many kits exist which allow for the detection of pre-formed staphylococcal enterotoxins. However, in many cases sample extraction is required prior to testing to produce a sample able to be analysed by the kits. Possibly the best available option for small laboratories is a latex agglutination kit, but the time required for a result is 20-24 hours, and there may be some problems with the methods which can only be overcome by sample preparation methods using reagents not supplied by the manufacturer. A kit for staphylococcal enterotoxin type B has been developed in the USA for biodefence purposes and this form of test could offer a solution. However, the kit has not been tested with foods and does not detect the enterotoxin with the greatest relevance to food (which is type A).

A promising approach would be to measure/detect the enzyme thermonuclease which is produced by coagulase positive staphylococci. Although a suitable kit does not currently exist, a method has been described which could be adapted. The method would need some development to establish a protocol and to simplify sample preparation. Additionally, a commercial source of the agar would need to be identified.

A review of the methods used by IANZ accredited laboratories show them to be following accepted mainstream methodologies, although it was not possible to assess one in house method. A full list of NZFSA approved dairy test methods is located on the NZFSA website http://www.nzfsa.govt.nz/registers-lists/approved-test-methods/.

At this time there are not available simple, cost-effective analytical methods that could be used in-house for monitoring *S. aureus* or it's enterotoxins by small businesses producing raw milk cheeses.

2 INTRODUCTION

Recent regulatory changes opened the way for domestic production of cheeses from raw milk where manufacturers are able to ensure product safety through a validated process, and demonstrate compliance under an approved Risk Management Programme. Clause 23 of the Animal Products (Raw Milk Products Specifications) Notice 2009 outlines the Food Safety Criteria applicable to such products including a requirement for absence of staphylococcal enterotoxin in 25g of product.

Staphylococcus aureus may occur in the milk of cows with clinical or sub-clinical mastitis or as the result of contamination by handlers. When toxigenic strains of this organism replicate to numbers exceeding 10^5 CFU/ml, they may produce staphylococcal enterotoxins.

While *Staphylococcus* is eliminated by pasteurisation, raw milk cheeses have no such pathogen elimination step. For this reason, the safety of raw milk cheeses is contingent upon monitoring of herds, temperature control of milk, and the cheese production and maturation steps themselves. It is essential that manufacturers of raw milk products can monitor the effectiveness of their process control steps in controlling bacterial numbers and are also able to undertake checks for the presence of enterotoxin for verification purposes and/or when process controls are outside of recommended limits.

Many food businesses that have expressed an interest in raw milk cheese production are small and lack microbiology resources and expertise. External laboratory fees are considered expensive. Ensuring access to information on options for process monitoring and routine in-house testing will form a useful resource for these manufacturers.

Identifying simple, cost-effective analytical methods for non-regulatory in-house testing would be of great benefit to this sector.

The objectives agreed with the NZFSA with respect to this project were:

- "To review available methods for detection/enumeration of coagulase positive *Staphylococcus* in terms of suitability for use in a milk/cheese matrix, sensitivity and specificity, rapidity, facilities required (lab or plant) and cost.
- To review available methods for detection of staphylococcal enterotoxin in terms of suitability for use in a milk/cheese matrix, sensitivity and specificity, rapidity, facilities required (lab or plant) and cost.
- To identify whether current testing methods are adequate for both regulatory testing and in-house industry monitoring "

These were to be achieved using the following methodology:

"Undertake a review of *Staphylococcus* tests and staphylococcal enterotoxin tests to evaluate:

- Which methods are routinely used in local labs
- What methods, kits and rapid tests are available, have they been validated for dairy products
- Whether methods identified are likely to be useful for process control in milk/cheese production

- Whether appropriate/useful/accurate methods are available for detection and quantification of staphylococcal enterotoxins, and have they been validated for dairy products"

Unfortunately the organism responsible for causing disease cannot simply be defined. References are made to "coagulase-producing *Staphylococcus aureus*" but it is known that some coagulase-producing strains do not produce enterotoxins, while the reverse is also true. Three other *Staphylococcus* spp. are also capable of producing coagulase, but while other staphylococci are also able to produce enterotoxins, it is almost always the case that foodborne intoxications are caused by *S. aureus* (Seo and Bohach 2007). Another characteristic of *S. aureus* is the production of a thermonuclease, or thermostable nuclease (Ibrahim 1981). In general, however, staphylococci that produce enterotoxins are coagulase and/or thermonuclease positive. The tests described below may therefore target different taxonomic groups, e.g. *S. aureus* specifically or the wider group of coagulase-producing staphylococci (CPS). In some cases it appears that this distinction has not been made or fully understood.

3 METHODS FOR THE DETECTION AND ENUMERATION OF CPS

A summary of methods for the enumeration of *S. aureus*/CPS is shown in Table 1. Given that the concentration at which CPS become a concern is quite high the detection limits of these tests do not need to be very low. As an example, in a validation of the ISO method the sample contaminated at a "low" level contained 10^3 CFU /g CPS (De Buyser *et al.* 2003). A standard microbiological procedure using agar plates, whereby 0.1 ml of a 1/10 diluted food sample is plated, gives a theoretical detection limit of 100 CFU/g if one colony is detected. This can be improved by using more plates, or plating a larger volume of food suspension. The ISO method Government of Ottawa method gives methods for detection limits of <25 CFU /g and <5 /g by varying volumes and the number of plates (www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mfhpb21-eng.pdf)

The "gold standard" for the enumeration of coagulase positive staphylococci in dairy products is based on the use of Baird-Parker agar (BPA) (Baird-Parker 1962) and this is one of the media used in the ISO 6888-1 method for coagulase-positive staphylococci (*Staphylococcus aureus* and other species).

S. aureus is the only coagulase and thermonuclease-producing *Staphylococcus* which is also capable of reducing tellurite and so this latter reaction is used in both selective agars (Baird-Parker 1962) and broths (Giolitti and Cantoni 1966). *S. aureus* also produces a lipase (lecithinase) which forms grey to black colonies with characteristic zones of clearing on BPA which contains egg yolk.

Baird Parker agar contains:

Pancreatic digest of casein	10.0g
Yeast extract	1.0g
Meat extract	5.0g
Sodium pyruvate	10.0g
Glycine	12.0g
Lithium chloride	5.0g
Agar	12.0-22.0g
Water	950 ml

The medium is completed by adding 50 ml of a 1% egg yolk emulsion and 10 ml of 1% potassium tellurite solution after autoclaving (sterilisation). The medium is available as pre-poured plates and so a small laboratory equipped with an incubator and pipettes could use the medium (more equipment may be needed for sample preparation)

A medium which may perform better than BPA is rabbit plasma fibrinogen agar (De Buyser *et al.* 1998). This is a Baird-Parker base (minus egg yolk) plus a supplement containing fibrinogen, rabbit plasma, trypsin inhibitor and potassium tellurite. Presumptive coagulase-producing staphylococci are grey or black, and surrounded by an opaque halo of precipitation which results from the coagulase reaction. When compared with BPA this medium was found to be less prone to overgrowth by competitive microbiota (De Buyser *et al.* 1998). It also incorporates the coagulase reaction so avoiding the need for further confirmation of colonies. A disadvantage is that the enumeration is performed as a pour plate, rather than a spread plate, adding a complicating step to the procedure and disallowing the purchase of pre-poured plates.

A comparison of the Bacteriological Analytical Manual and Petrifilm™ Staph Express methods showed them to produce similar results (Fedio *et al.* 2008) with both methods able to detect 10-100 CFU /g *S. aureus* in 50g samples of six cheese types. The background flora did not interfere with detection. Similarly, another comparison of BPA and Petrifilm™ Staph Express showed consistent results when cheese was inoculated with 100-100 CFU /g. The validation of RAPID'*Staph* agar reported that non-*S. aureus* bacteria either did not grow on the medium or would not be mistaken for the pathogen (Lauer *et al.* 2007). In milk *S. aureus* could be enumerated at concentrations of 20-50 CFU /g.

There are very many published PCR-based methods (Cremonesi *et al.* 2007) and no attempt has been made to provide comprehensive coverage of them since any PCR-based system requires molecular biology skills, the purchase of specific equipment and the implementation of excellent laboratory procedures to prevent cross contamination. PCR-based methods are subject to interference and inhibition from food components, hence requiring DNA isolation and purification which adds to the cost of the test. There is also some question as to whether the detection of a gene reflects the ability of the organism to express it either at all, or sufficiently, such that enough SE is formed to cause disease. As an example, 33 raw milk cheeses were found to contain no toxin as judged by the use of immunological assays but 13 of 14 samples contained *se* genes (Scientific Committeee on Veterinary Measures Relating to Public Health 2003). An advantage of PCR is that, given suitable sequence data, PCR detection of newly identified genes is possible in a short time scale; immunological methods may take longer to develop. Reverse-transcriptase PCR might also be used to measure the expression of SE genes. "Research" PCR methods can have a detection limit of 100 CFU/g (Cremonesi *et al.* 2007) to 55 CFU /g (Yang *et al.* 2007), and good primer design should ensure that there are no false positive detections given these caveats.

Most of the PCR methods could be regarded as being for "research" and so have not been summarised here, but commercial systems are available, for example the BAX® real time PCR system http://www2.dupont.com/Qualicon/en_US/assets/downloads/BAX%20product%20descrip-RealTime%20Staph.pdf). See Table 2 and the Appendix for more overview information, including sensitivity data. The requirement to purchase or lease the appropriate PCR machine means that laboratories with high throughputs are likely to be those for which this system is economically viable.

Other kits (Table 2) using genetic approaches rely on hybridization. However, they also require a well-equipped laboratory in order for them to be implemented.

Many slide agglutination assays for the confirmation of CPS (although the kits tend to claim that they identify *S. aureus*) are available and undoubtedly more are available than indicated in Table 1. These tests generally use two characteristics. The first is the possession of a cell-bound coagulase, or "clumping factor". The second is the possession by 90% of *S. aureus* isolates of protein A on their cell surface which has an affinity to Immunoglobulin G. Beads coated with IgG therefore clump together in the presence of *S. aureus*. Possession of either of these characteristics will yield a positive slide agglutination test. These kits would be ideal for use in small laboratories as they are easy to perform, rapid and easy to interpret.

An alternative approach to the detection of CPS may be to measure the presence of thermonuclease as suggested in an EFSA report (Scientific Committee on Veterinary Measures Relating to Public Health 2003) and proposed previously (Tatini *et al.* 1975). While the detection of this enzyme is reasonably straightforward for a scientific laboratory (Hamama *et al.* 2002) it requires a refrigerated centrifuge, a boiling waterbath, specialized agar plates and a 50°C incubator. No rapid method or kit form of this analysis could be located.

A "simple sensitive" method for demonstrating thermonuclease activity has been described (Ibrahim 1981). This involves some sample preparation but if the agar were to become commercially available through demand then this represents a possible method that could be adapted to be easier to perform. A 20 g sample of cheese is blended with 30 ml warm water, and heated at 100°C for 20 minutes. The sample is centrifuged (27,000 x g) for 20 min and the supernatant used for testing (although perhaps a filtration step would be more practical). Wells are formed in agar plates of agar containing DNA and a dye, 2 x 30 µl of the supernatant added and the plates incubated at 50°C for 4 hours. Bright pink zones show thermonuclease.

In addition to the following Tables, a full list of NZFSA approved dairy test methods is located on the NZFSA website.

http://www.nzfsa.govt.nz/registers-lists/approved-test-methods/

Table 1 Conventional and rapid methods for the enumeration/detection of *S. aureus*/CPS in foods

Test kit/method name and type	Manufacturer	Catalogue Number	Foods covered	Validation	Comments and cost	Equipment
BBL™CHROMagar™ Staph aureus Agar medium	BD Diagnostics	214982, 252715, 257074, 257099	Cooked roast beef, smoked salmon, shell eggs	AOAC performance tested. Detects and enumerates *S. aureus*.	Available RTU. $30 per 10 plates. Further information in Appendix.	Fully equipped microbiology laboratory
RAPID' Staph Agar Agar medium	Bio-Rad Laboratories	356-3960 (ready to use); 356-4704 (500g dehydrated)	Pasteurised whole milk, custard pie, processed ham and smoked salmon	AOAC performance tested, AFNOR (BRD-07/09-02/05).(Lauer *et al.* 2007). Enumerates CPS.	Price not supplied by agent. Further information in Appendix.	Fully equipped microbiology laboratory
TECRA Staphylococcus aureus VIA™ (NZTM2.47.3) ELISA	3M Microbiology	STAVIA96	-	NZFSA. Detects *S. aureus*.	Sensitivity 1-5 cfu/3g food. $1063.15/96 well kit. Further information in Appendix.	Incubator, blender/homogeniser
3M™ Petrifilm™ Staph Express Count Plates Thin film medium	http://solutions.3m.com/wps/portal/3M/en_US/Microbiology/FoodSafety/	6490 to 6493	Selected dairy foods (ice cream, raw milk, yoghurt, whey powder, cheese)	Numerous, including AFNOR (3M-01/09-04/03A) , AOAC first action in selected dairy foods (Silbernagel *et al.* 2003), New Zealand NZFSA, NordVal Enumerates *S. aureus*.	$1,954.45 for 500 tests (no spreader), 100 confirmation discs $383.40. Further information in Appendix.	Incubator, blender/homogeniser
Staphylococcus aureus (enumeration) by Nutricia Method M01_05ME	-	-	-	NZFSA approved	No information could be located, so in house method likely	
EN ISO 6888-1, 2:1999 Culture	N/A	N/A	Cheese, meat, dried egg powder	De Buyser et al., 2003. Detects CPS.	N/A	Fully equipped microbiology laboratory
NZTM 2.47.1 Coagulase-positive Staphylococci; Count Issue 13.2: March 2007 Culture	N/A	N/A	All types of milk and milk products	NZFSA. Detects CPS	N/A	Fully equipped microbiology laboratory
NZTM 2.47.2 Coagulase - positive Staphylococci; Detection Issue 16.0: August 2009 Culture	N/A	N/A	All types of milk and milk products	NZFSA. Detects CPS	Uses (Lancette and Lanier 1987) broth	Fully equipped microbiology laboratory
MPN method Culture	N/A	N/A	Milk, tuna salad, minced turkey	AOAC 46.062. (Lancette and Lanier 1987). Enumerates *S. aureus*.	Current?	Fully equipped microbiology laboratory
NEO-GRID™ / ISO-GRID™ Hydrophobic grid membrane filtration method.	Neogen	Multiple; this is a system.		Claims to enumerate *S. aureus*, but uses BP so probably detects CPS.	Further information in Appendix.	Fully equipped microbiology laboratory

Test kit/method name and type	Manufacturer	Catalogue Number	Foods covered	Validation	Comments and cost	Equipment
Tube coagulase test Culture	N/A	N/A	Milk	(Yazdankhah and Olsen 1998). Claims to detect *S. aureus*, probably detects CPS.	Research method	"of use to..with limited laboratory facilities, or to dairy farmers as a simple diagnostic test on site"
Baird Parker agar Agar medium	Numerous vendors	-	N/A	(De Buyser *et al.* 2003) $14 per sleeve of 20 plates. Detects CPS.	The "gold standard" agar. Used in ISO method. Available RTU	Available as pre-poured plates.
Soleris™ Culture	Neogen	BS-128 (128 system without computer)	NS	None found. Detects *Staphylococcus*.	Uses change in pH of selective media. $3.80 per test, positives need to be confirmed. Further information in Appendix.	Specialised equipment needs to be purchased
Rabbit plasma fibrinogen agar Agar medium	Oxoid	SR122 plus supplement	N/A	(De Buyser *et al.* 1998). NMKL Method No. 66 (Baird parker + rabbit fibrinogen plasma supplement). Detects CPS.	Used in ISO method, ranks equal with Baird Parker. About 5 times more expensive than BPA (Chang and Huang 1996) but no confirmation needed.	Cannot be bought as pre-poured plates.
BBL™ Staphyloslide™ Latex Test Kit Latex agglutination	BD Diagnostics	FTR240952 (Fort Richard)	N/A	Detects staphylococci which are clumping factor (bound coagulase) and/or protein A positive.	For identification of isolated colonies. $290.53 for 100 reactions. Further information in Appendix.	
AureusTest Latex agglutination	Trisum Corp. Taipei, Taiwan		N/A	AOAC 995.12		
STAPHYTECT PLUS Latex agglutination	Oxoid	Code: DR0850	N/A	(Fujikawa and Igarashi 1988)	For identification of isolated colonies	
Mircogen Staph Latex Latex agglutination	Microgen		N/A		For identification of isolated colonies. $410 AUD for 500 tests +$35 AUD for control reagent. Further information in Appendix.	
ANI S. aureus Latex agglutination	ANI Biotech, Finland		N/A	Detects staphylococci which are clumping factor (bound coagulase) and/or protein A positive.	For identification of isolated colonies. Further information in Appendix.	
Phadebact® Staph Aureus test Latex agglutination	Bactus AB, Sweden		N/A		For identification of isolated colonies	
Pastorex Staph plus Latex agglutination	BIO-RAD	56356	N/A	(Compernolle *et al.* 2007)	For identification of isolated colonies. Further information in Appendix.	
Staphylase Latex agglutination	Oxoid	OXODR0595A	N/A		For identification of isolated colonies. $238.70 for 1000 eactions.	

Test kit/method name and type	Manufacturer	Catalogue Number	Foods covered	Validation	Comments and cost	Equipment
Staphaurex® Latex agglutination	Remel	R30859901	N/A	Evaluated in (Berke and Tilton 1986), the joint most sensitive method. No negative control.	For identification of isolated colonies	
Staphaurex® Plus Latex agglutination	Remel	LAG30950102		Detects staphylococci which are clumping factor (bound coagulase) and/or protein A positive. (Wilkerson *et al.* 1997)	For identification of isolated colonies. $309 for 150 reactions.	
Hemastaph Latex agglutination	Remel		Suspect discontinued-not located on Remel website.	Evaluated in (Berke and Tilton 1986).	For identification of isolated colonies	
Staphyloslide Latex agglutination	BBL Microbiology systems			Evaluated in (Berke and Tilton 1986). Detects staphylococci which are clumping factor (bound coagulase) and/or protein A positive.	For identification of isolated colonies	
Veri-Staph Latex agglutination	Zeus technologies		Possibly discontinued.	Evaluated in (Berke and Tilton 1986) and found to be the poorest performer with respect to specificity and highest with respect to sensitivity.	For identification of isolated colonies	
BactoStaph Latex agglutination	Difco Laboratories			Evaluated in (Berke and Tilton 1986). Lowest sensitivity.	For identification of isolated colonies	
FastStaph Latex agglutination	Carr-Scarborough Microbiological			(Wilkerson *et al.* 1997)	For identification of isolated colonies	
Slidex Staph kit Latex agglutination	BioMérieux			(Wilkerson *et al.* 1997)	For identification of isolated colonies	
SeroSTAT Latex agglutination	Scott Laboratories			Evaluated in (Berke and Tilton 1986). Hardest to read.	For identification of isolated colonies	
MASTASTAPH Latex agglutination	MAST	RST 101		Detects *S. aureus*.	For identification of isolated colonies	

RTU = ready to use. AOAC = Association of Official Analytical Chemists. AFNOR = Association Française de Normalisation NZTM = New Zealand Technical Manual, ELISA = Enzyme Linked Immunosorbent Assay.
NB. The prices quoted are for the kit/product alone. There will be labour costs and other consumables involved in using them…

Table 2 Genetic methods for the Detection/Identification of *S. aureus*/CPS

Test kit name	Manufacturer	Catalogue Number	Foods covered	Validation/approvals	Comment/cost	Equipment requirement
BAX® System Real-Time PCR Assay for *Staphylococcus aureus*	Dupont Qualicon, Inc.	D12762689	Ground beef, soy protein isolate, and soy and milk based powdered infant formula	AOAC performance tested	Presence absence testing and threshold testing for minced beef. Detects *S. aureus*. Cost approx. $90,000 + GST for the equipment. A kit costs approx $1800 for 96 tests. Further information in Appendix.	PCR machine needs to be purchased. Price not supplied by agent. Dedicated laboratory area required.
GENE-TRAK Based on DNA hybridisation.	Neogen		Not stated	None found	Company states detects 3 cfu/g. Test over in 3h (post-enrichment). $990/98 tests (some of which would be controls). Detects *S. aureus*.	Needs a photometer, and two waterbaths.
Accuprobe Based on DNA hybridisation	Gen-Probe	2875	Not stated-use on isolates only	Canadian government MFLP-79	For culture confirmation. Detects *S. aureus*. Further information in Appendix.	Needs a luminometer
Genevision Real time PCR assay.	Warnex	Laval, QC, Canada	Not stated	Canadian government (Ottawa)	Can be used in a semi-quantitative mode (i.e. if the conc of staph exceeds some threshold). No examples of this being used in the literature could be located.	RT PCR machine

NB. The prices quoted are for the kit/product alone. There will be labour costs and other consumables involved in using them.

4 METHODS FOR THE DETECTION AND MEASURMENT OF STAPHYLOCOCCAL ENTEROTOXINS

Given that many staphylococcal enterotoxins (SEs) have now been identified (21 to date (Derzelle *et al.* 2009)) and that no rapid method is able to detect them all it is fortunate that only a few are of significance. These "classically described" enterotoxins are SEA, SEB, SEC, SED, and SEE, but most foodborne intoxications occur following the consumption of staphylococcal enterotoxin SEA (Stewart 2003). Some very large outbreaks have also been attributed to SEA consumption (Asao *et al.* 2003).

Conventional methods for detecting the presence of SEs are complex (Lancette and Bennett 2001), making them impractical for all but the best-equipped laboratories. Therefore the rapid methods, which generally come in kit form, are the only practical options for routine testing laboratories. These range from fully automated systems requiring significant capital outlay to plate ELISA systems which are relatively cheap to use. However, the minimum equipment required by a laboratory is a centrifuge and an incubator, as well as the facilities to process the food samples. The manufacturers of ELISA systems, while stating that the results may be read visually, recommend the use of specific detection equipment to quantify colorimetric results.

A table showing details of the equipment that was located in a search is given in Table 3. The widest range of SEs detected is A to E and some accommodate a more limited range (A-D).

None of the methods listed would appear to be ideal for in-house use by a small/medium sized manufacturer. The methods are either very expensive requiring initial specialized equipment to be bought (e.g. the VIDAS system), or require a well-equipped laboratory (e.g. TECRA).

Questions have been raised about the sensitivity of some of these kits (Scientific Committee on Veterinary Measures Relating to Public Health 2003). However, it is also not clear what quantity of toxin is required to produce illness although phrases such as "1 ng/g of food or less" are frequently used in the literature. The ELISA kits have detection limits at around this concentration (i.e. 0.5 - 1 ng/g), although this is dependent on the dilution that occurs during toxin extraction.

Nevertheless, notwithstanding any uncertainty over the concentration of enterotoxin required to cause illness, clause 23 of the Animal Products (Raw Milk Products Specifications) Notice 2009 states a regulatory requirement for absence of staphylococcal enterotoxin in 25g of product.

A comparison of VIDAS, TECRA and TRANSIA tube methods showed them to be of broadly similar sensitivity (Bennett 2005) although the VIDAS method was superior to the others at detecting renatured toxin in naturally contaminated canned mushrooms. TRANSIA was shown to be more sensitive than RIDASCREEN for the detection of SEB in egg custard (Schotte *et al.* 2002). TECRA kits may be affected by non-specific reactions which can be overcome by adapting sample preparation (Park *et al.* 1992). The TRANSIA and VIDAS SET2 methods also need special sample preparation when used to test cheese (Hennekinne *et al.* 2007a, Lapeyre *et al.* 1996).

The sensitivity of the kits can be increased by concentrating the sample after extraction although this raises one more hurdle to the implementation of such methods in small laboratories or small businesses.

Possibly the best available option for small laboratories is a latex agglutination kit, but the time required for a result is 20-24 h. The Oxoid kit has been shown to detect toxin (type unreported) in Halloumi cheese (Berry *et al.* 1987) although the same paper also reported a non-specific autoagglutination in sheep's milk cheese. It has also been used to detect SEC in Moroccan traditional cheese (Hamama *et al.* 1992) and a variety of SE types in "herby" cheese (Akkaya and Sancak 2007). The problem of non-specific reactions with cheese samples has been addressed and the addition of 10 mM hexametaphosphate to the diluent was shown to reduce non-specifc reactions in most cases (Rose *et al.* 1989). This non-specific reaction was reported to be associated with renneted cheeses (but not all of them) and the diluent used. The sensitivity of the kit was determined to be 0.25 ng/ml extract.

A test strip detecting SEB produced for biodefence applications has been described (Schotte *et al.* 2002). The strip, which is designed in a "pregnancy test" format performed better than either RIDASCREEN or TRANSIA ELISA methods. A "similar" product is available from a US defence company (www.alexeter.com). This format of kit would be suitable for use in small laboratories, but it only detects SEB and was designed to detect SEB in powders and not in foods.

Other approaches do exist but tend to be applicable only to laboratories operating sophisticated equipment. Form example, liquid chromatography mass spectrometry has been used to measure SEB (Callahan *et al.* 2006, Seto and Kanamori-Kataoka 2005).

Very recent press reports (e.g. http://www.foodqualitynews.com/Publications/Food-Beverage-Nutrition/FoodProductionDaily.com/Quality-Safety/Breakthrough-test-for-food-poisoning-bug/?c=VAVnu3Gw58OSK0Yn5qCI6g%3D%3D&utm_source=newsletter_daily&utm_medium=email&utm_campaign=Newsletter%2BDaily) state that the USDA have produced a new sensitive method for the detection of SEA. However, given that "The assay neatly exploits this trait by measuring proliferation of splenocytes, which are immune system cells produced in the spleen," said the ARS statement. "For the assay, the cells are kept alive in laboratory petri dishes"" this approach does not seem to be useful for small laboratories. No scientific information on the method or validating it could be located.

Table 3 "Rapid" or kit methods for SE detection

Test kit/method name	Manufacturer	Catalogue Number	Analyte (SEs)	Foods covered	Validation/approvals	Detection limit *	Comment /cost	Equipment required
VIDAS® Staph enterotoxin (SET) Immunoassay	BioMérieux					<0.5 ng/g SEA and SEC2, >1 ng/g SED and SEE (Vernozy-Rozand *et al.* 2004)	Superceded by SET 2	High capital outlay
VIDAS® Staph enterotoxin II (SET2) Immunoassay	BioMérieux. http://www.biomerieux.com.au/servlet/srt/bio/australia/dynPage?doc=AST_IND_FDA_PRD_G_PRD_NDY_7	30 705	A, B, C (C1, C2, C3), D and E	Variety of Foods	AOAC performance tested. Accepted as a CRL method alongside Transia® kit. (Bennett 2005) (Hennekinne *et al.* 2007a)	<0.5 ng/g A and B, <1 ng/g C2 and E, 1 ng/g D (Vernozy-Rozand *et al.* 2004)	Better than Transia® (Hennekinne *et al.* 2007b),(Vernozy-Rozand *et al.* 2004). Better than VIDAS SET (Vernozy-Rozand *et al.* 2004)	High capital outlay
TECRA Staphylococcal enterotoxin VIA™ ELISA	3M Microbiology	SETVIA48, SETVIA96	A, B, C1, C2, C3, D and E	Canned mushrooms, nonfat dry milk, canned lobster bisque, beef and pasta, cooked chicken (Bennett and McLure 1994). More, including cheese (Bennett 2005).	AOAC Method No. 993.06 Bennett and McClure 1994, NZDB (New Zealand Dairy Board), MAB (Ministry of Agriculture, Brazil), Canadian government MFLP-68	1 ng/ml	Cross reactions i.e. false positives, reported ((Park *et al.* 1992), Park et al 1994). Complete within 4h (Su and Wong, 1997). May require sample concentration, which adds more complicated laboratory steps	Standard lab equipment plus incubator, centrifuge. ELISA plate reader recommended (as otherwise colour judged visually)
European screening method of the Community Reference Laboratory (CRL) Enzyme immunoassay.	N/A, uses Transia® method	N/A	A-E	Cheese	(Hennekinne *et al.* 2003)		A laborious method requiring dialysis concentration prior to testing.	Fully equipped laboratory
RIDASCREEN Immunoassay	r-Biopharm, Darmstadt, Germany	R4101	A-E	Various including cheese	(Park *et al.* 1994, Park *et al.* 1996), Canadian government MFLP-69	Depending on the tested food sample: 0.2 - 0.7 ppb. 0.2-0.75 ng/ml extract	<3 h to complete, no complex extraction	Spectrophotometric measurement

Transia® (Transiatube and TransiaPlate) Immunoaffinity, ELISA	Diffchamb, Lyon, France	ST0796	A-E	Milk and dairy products	CRL reference method. (Bennett 2005) (tube assay)	0.1 ng/g sample according to manufacturer, reported less than this (Vernozy-Rozand *et al.* 2004)	CRL reference method. Uses a non-dialysis method for concentration. No negative control (Su and Wong 1997)	
SET RPLA	Oxoid	TD 0900	A-D	"a wide variety"	Canadian government MFLP-67. (Berry *et al.* 1987)	0.5 ng/ml of extract, so limit in food will vary by the preparation needed. A 1:1 dilution will give 1 ng/g food	20-24 h for the reaction to occur.	Needs a centrifuge for sample preparation
SET-RPLA "SEIKEN" RPLA	Denka Seiken	230829	A-D				Same as Oxoid product (Berry *et al.* 1987, Fujikawa and Igarashi 1988). Not as sensitive as SET-EIA (Berry *et al.* 1987)	
Bio Threat Alert™ Immunochromatography	ww.alexeter.com		B	-	(Schotte *et al.* 2002) for a "similar" product	0.0625 ng for a "similar" product	45 minutes for sample preparation and testing. Intended for testing powders suspected as being involved in terrorism.	Would need a 20µl pipette and a means to prepare the sample.
3M™ Tecra™ SET ID VIA ELISA	3M Microbiology	SID VIA 72	A-E				For toxin identification following positive ELISA	
SET-EIA Immunoassay	Labor Dr. Bommeli AG, Switzerland. Now owned by IDEXX		A-D		(Berry *et al.* 1987, Wieneke 1991)		Most sensitive kit and so no concentration required. Labour intensive and 20h needed for a result (Su and Wong 1997)	

4 ng/g food has been recommended as the sensitivity required (Bergdoll 1991) although others state 1ng/g of food .
NB. The prices quoted are for the kit/product alone. There will be labour costs and other consumables involved in using them.

5 METHODS USED IN NEW ZEALAND LABORATORIES

Table 4 shows the laboratories listed on the IANZ website (http://cabis.ianz.govt.nz/ianzwebportal/) with accreditation for the detection and/or enumeration of *S. aureus* and/or its toxin. There is a good degree of agreement over the methods used in that the predominant approach is to use the ISO method, albeit with some variations.

Comment cannot be made concerning the Nutricia and Nestle methods, other than to say that the Nutricia method has been accepted by the NZFSA. The details do not seem to be publicly available. All of the other methods used appear to be quite satisfactory in that they are primarily based on ISO, APHA or BAM methods. These are, in general, based around the use of BPA which remains the "gold standard". The selection of laboratories testing for toxin is limited.

Table 4 Methods use in New Zealand for analysis of *S. aureus*/CPS/SE in dairy products

Laboratory Name	Location	Food	Test	Method
AsureQuality	Auckland	Milk, cream, milk powders, butter, other fat products, cheese, caseins and caseinates, whey products, cultured products, frozen confectionery, dairy foods, ice cream, alamin, lactose, milk protein concentrate	Coagulase-positive *Staphylococcus aureus*	NZTM 47.1, NZTM 47.2, ISO 5944/IDF 60:2001, ISO 6888-1:2003
			Staphylococcus aureus count	132.89
			Staphylococcal enterotoxin	FDA BAM Ch.13A (ELISA)
		Milk powder	*Staphylococcus aureus* count	Nutricia M01_05ME:2001
		Dairy products	*Staphylococcus aureus*	BAM 12
			Staphylococcal enterotoxin	TECRA ELISA
	Lower Hutt	Cheese	Coagulase-positive Staphylococci (Count)	NZTM 47.1
	Christchurch	Dairy products	*Staphylococcus aureus*	BAM Ch. 12
		Milk, cream, milk powders, butter,	Coagulase-positive Staphylococci (Count)	NZTM 47.1

Laboratory Name	Location	Food	Test	Method
		other fat products, cheese, caseins and caseinates, whey products, frozen confectionery, dairy foods, ice cream, milk protein concentrate	Coagulase-positive Staphylococci (Detection)	NZTM 47.2
Cawthron	Nelson	Milk, cheese, ice cream	Coagulase-positive Staphylococci (Count) Coagulase-positive Staphylococci (Detection) Staphylococcus aureus	ISO 6888-1:1999 (Amendment 2003) NZTM 47.2 AOAC 2003.08 (Petrifilm)
Environmental Laboratory Services	Lower Hutt	Milk, milk powders, chees, whey products, cultured foods, dairy foods Dairy products	Coagulase-positive Staphylococcus aureus Staphylococcus aureus	IDF 145A:1997 FDA BAM Ch.12 (online)
ESR	Christchurch	Dairy products	Coagulase positive Staphylococcus Staphylococcus enterotoxin	APHA 4th edidtion 39 TECRA ELISA
Fonterra Research Centre, Microbial Fermentation Unit	Palmerston North	Dairy starter cultures	Coagulase positive Staphylococcus aureus	NZTM2:47.2 (modified)
Fonterra Ltd	Hawera	Milk powders, milk protein concentrate	Coagulase-positive Staphylococci (Count)	NZTM 47.1

Laboratory Name	Location	Food	Test	Method
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2 (modified)
			Coagulase-positive Staphylococcus aureus	NZTM 47.3 (Visual Immunoassay)
		Butter	Coagulase-positive Staphylococci (Count)	NZTM 47.1
		Cheese, caseins and caseinates, whey products, alamin, lactose	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
		Alamin, Lactose	Coagulase-positive Staphylococcus aureus	NZTM 47.3 (Visual Immunoassay)
	Hamilton	Milk, milk powders, butter, other fat products, cheese, caseins (lactic, rennet, TMP, caseinates), whey protein concentrate, lactose, milk protein concentrate, Lactalbumin	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
			Coagulase-positive Staphylococcus aureus visual immunoassay modified	NZTM 47.3 (automated/manual)

Laboratory Name	Location	Food	Test	Method
	Temuka	Milk powder	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
			Coagulase-positive Staphylococcus aureus	NZTM 47.3 (visual immunoassay)
		Cheese	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
		Caseins and caseinates	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
			Coagulase-positive Staphylococcus aureus	NZTM 47.3 (visual immunoassay)
			Coagulase-positive Staphylococcus aureus	NZTM 130.12
		Alamin	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
		Lactose	Coagulase-positive Staphylococci (Count)	NZTM 47.1
			Coagulase-positive Staphylococci (Detection)	NZTM 47.2
			Coagulase-positive Staphylococcus aureus	NZTM 47.3 (visual immunoassay)
		Milk protein concentrate and Milk	Coagulase-positive Staphylococci (Count)	NZTM 47.1

Laboratory Name	Location	Food	Test	Method
		protein isolate	Coagulase-positive Staphylococci (Detection)	NZTM 47.2
Kapiti Fine Foods Limited	Palmerston North	Milk, cream, ice cream	Staphylococcus aureus Petrifilm Staph Express	NZTM2:63.4
Hill Laboratories	Christchurch	Dairy products	Staphylococcus aureus	APHA 4th edition 39.5, FDA BAM Chapter 12
Heinz Wattie Limited	Hastings	Dairy products (frozen dairy-based sauces)	Coagulase positive Staphylococcus	APHA 4th edition 39, NZTM 2.47.1
Nestle New Zealand Limited	Auckland	Dairy products	Staphylococcus aureus	Nestle International Company methods
New Zealand Laboratory Services Limited	Auckland and Christchurch	Dairy products	Coagulase positive Staphylococcus	APHA 4th edition 39, AS 1766:2.4, ISO 6888.1:1999 (E)
	Hastings	Dairy products (ice cream)	Staphylococcus aureus	APHA 4th edition, 39.53
	Auckland	Milk, cream, milk powders, butter, other fat products, cheese, caseins and caseinates, whey products, cultured products, frozen confectionery, dairy foods, ice cream, rennet, alamin	Coagulase-positive Staphylococci (Count)	NZTM 47.1, NZTM 47.1 (modified), Nutricia
			Coagulase-positive	47.2 (modified), Nutricia

Laboratory Name	Location	Food	Test	Method
			Staphylococci (Detection)	
			Coagulase-positive Staphylococci	ISO 6888-1:1999, ISO 5944, IDF 60 (modified)
	Hastings	Milk, butter, cheese	Staphylococcus aureus (Count)	NZTM 47.1
	Hamilton	Dairy products	Staphylococcus aureus	APHA 4th edition, 39
Nutricia New Zealand Laboratories	Auckland	Milk powders	Staphylococcus aureus (Count)	MTM 15.0 (Nutricia method)
Puhoi Valley Cheese	Auckland	Cheese	Staphylococcus aureus (Petrifilm)	AOAC 2003.08
SGS Food and Environment Laboratories Limited	Auckland and Christchurch	Dairy products	Staphylococcus aureus	APHA 4th edition, 39
Tatua Co-operative Dairy Company Ltd	Morrinsville	Other specified products (cream/mousse)	Coagulase-positive Staphylococcus aureus	IDF 60C:2001 (modified)
		Whey protein concentrate	Coagulase-positive Staphylococcus aureus (Detection)	IDF 60C:2001 (modified)
			Coagulase-positive Staphylococcus aureus (Count)	IDF 145A:1997
		Caseins and caseinates	Coagulase-positive Staphylococcus aureus (Detection)	IDF 60C:2001 (modified)
			Coagulase-positive	IDF 145A:1997

Laboratory Name	Location	Food	Test	Method
			Staphylococcus aureus (Count)	

ACRONYMS: IDF International Dairy Federation, American Public Health Association, AOAC (formerly) the American Association of Official Analytical Chemists, ISO International Standards Organisation, NZTM: New Zealand Technical manual, FDA BAM: Food and Drug Administration Bacteriological Analytical Manual

6 CONCLUSIONS

A plethora of methods is available for the detection of CPS and SEs. Conventional cultural methods based on Baird-Parker agar have now been used for decades and are still regarded as the "gold standard". They form the basis of widely used standard methods such as the ISO method and thus ideal for regulatory purposes. For small laboratories, however, these conventional methods would be difficult to follow given the need for expertise and special equipment. Rapid tests for the presence of CPS are relatively expensive to perform and there are other problems such as the need for sample preparation and the short shelf lives of kits once opened. The identification of colonies once isolated is less of a problem because there is a myriad of slide agglutination kits available for this purpose. Their isolation is, however, problematic.

More contemporary genetic-based (i.e. PCR or DNA hybridization) detection methods require large capital outlay or, again, a fully equipped laboratory staffed by people with an appropriate skill level to operate successfully. Manufacturers with sufficient throughput might find these to be useful, but with small manufacturers this is less likely to be so. These methods can be sensitive; the information for the BAX® system reproduced below indicates a sensitivity of 1 CFU/g in powdered infant formula (for which is has AOAC approval), while GENE-TRAK can detect 1-5 CFU/ 25g. Both of these sensitivities are achieved following enrichment. No papers describing their applications to cheese could be located.

Detection of toxin could be performed with minimal equipment if a RPLA method were to be adopted (given that ELISA kits would be technically too demanding for small laboratories and VIDAS too expensive). Questions remain in respect to the sensitivity of these systems and there is limited literature describing their application to dairy products. There is little in the way of regulatory approval around this sort of product. An alternative, which has been removed from the market, is the TECRA Unique system.

Of note is the development of "pregnancy test" format assays in terms of ease of use and sensitivity, but data showing their efficacy for food control purposes are missing from the literature, the kit only detects SEB and their availability needs to be established.

A promising approach would be to measure/detect thermonuclease but a suitable kit does not currently exist (to the best of my knowledge). A method that could be adapted is, however, discussed above (page 4). The method would need some work to establish a protocol and to simplify sample preparation, and a commercial source of the agar identified.

However, at this time there are not available simple, cost-effective analytical methods that could be used in-house for monitoring *S. aureus* or it's enterotoxins by small businesses producing raw milk cheeses. Small businesses need to factor in the costs of testing conducted by accredited laboratories.

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8 APPENDIX

The following links will provide additional information on the kits mentioned:

ACCUPROBE®

http://www.gen-probe.com/pdfs/pi/102944RevG.pdf

ANI™ *S. aureus* Test

http://www.anibiotech.fi/anibiotech/pdfs/Staph.pdf

BAX® System

http://www2.dupont.com/Qualicon/en_US/products/BAX_System/bax_realtime_staph.html

BBL™ CHROMagar™ Staph aureus

http://www.bd.com/ds/technicalCenter/promotionalFlyers/ss_222307.pdf

DENKA SEIKEN

http://www.denka-seiken.co.jp/english/products/bacteriology/staphylococcusAureus.html

GENE-TRAK Staphylococcus aureus Assay

http://www.tnb.co.th/testkits/staphy.html

MICROGEN® STAPH RAPID TEST

http://keydiagnostics.com.au/index.php?page=shop.product_details&flypage=shop.flypage&product_id=10&category_id=8&manufacturer_id=0&option=com_virtuemart&Itemid=26

NEO-GRID™ / ISO-GRID™

http://www.aqua-salis.dk/data/images/f%C3%B8devare/ISO-Grid_Staph.pdf

Pastorex™ Staph-Plus Kit

http://www.bio-rad.com/prd/en/US/adirect/biorad?cmd=BRCatgProductDetail&isFromSearch=true&entry

Point=adirect&catID=0a84ccab-dc48-45e9-bd12-b21792da97ae&vertical=CDG&messageType=BRCatgProductDetail&parentCategoryGUID=72ec6f8b-f937-4ed7-956b-23eac62f9fb8

3M Petrifilm

http://solutions.3m.com/wps/portal/3M/en_US/Microbiology/FoodSafety/product-information/product-catalog/?PC_7_RJH9U523003DC023S7P92O3O87_nid=D7BKZ3NP1Mbe29BDXSBJ7Fgl

RAPID'Staph

http://www3.bio-rad.com/B2B/BioRad/product/br_category.jsp?BV_SessionID=@@@@1615326454.1265944559@@@@&BV_EngineID=cccdadejilkihlecfngcfkmdhkkdfll.0&divName=Food+%7c+Animal+%7c+Environment+Testing&categoryPath=%2fCatalogs%2fFood+%7c+Animal+%7c+Environment+Testing%2fFood+Testing%2fChromogenic+Media+%2fRAPID+Staph+Medium&loggedIn=false&lang=English&catLevel=5&country=ZA&catOID=-32712&isPA=false&serviceLevel=Lit+Request#overview

RIDASCREEN

http://www.r-biopharm.com/product_site.php?product_id=250&product_class_one=TWljcm9iaW9sb2d5IC8gSHlnaWVuZQ==&product_class_two=U3RhcGh5bG9jb2NjYWwgZW50ZXJveGluIChTRVQp&product_class_three=&product_class_four=&product_range=Food%20and%20Feed%20Analysis&Food%20and%20Fe

http://www.r-biopharm.com/product_site.php?product_id=4477&product_class_one=TWljcm9iaW9sb2d5IC8gSHlnaWVuZQ==&product_class_two=U3RhcGh5bG9jb2NjYWwgZW50ZXJveGluIChTRVQp&product_class_three=&product_class_four=&product_range=Food%20and%20Feed%20Analysis&

SET-RPLA KIT TOXIN DETECTION KIT

http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=TD0900&c=UK&lang=EN

SOLERIS

http://www.biolabgroup.com/AUS/LAB/pdf/MicroBiol/Spoilage/Soleria_Staphylococcus.pdf

STAPHYLOCOCCUS AUREUS VISUAL IMMUNOASSAY (VIA™)

http://www.tecra.net/__data/page/61/STAVIAPI2.pdf

TECRA Staphylococcal Enterotoxin VIA™VISUAL

http://www.tecra.net/__data/page/60/SETVIApiNov05.pdf

BBL™ Staphyloslide™ Latex Test for Staphylococcus aureus

http://www.bd.com/ds/technicalCenter/clsi/clsi-staphyloslide.pdf

Transia

http://www.biocontrolsys.com/pdf/SL_SET(4).pdf