CURRENT DEVELOPMENTS IN QUANTIFICATION OF PATHOGENS FROM ENVIRONMENTAL MEDIA: THE TRADEOFF OF ACCURACY AND EASE IN THE CASE OF *SALMONELLA* IN BIOSOLIDS

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ABSTRACT

The quantification of *Salmonella* in biosolids is typically performed by culture-based techniques, which are time-consuming and vulnerable to many confounding problems such as the under-estimation of pathogen levels due to the presence of viable but non-culturable (VNBC) state cells. Molecular quantification techniques potentially offer much more rapid and reliable analysis. This study is aimed at adaptation and application of the most current molecular based techniques, Taqman assisted qPCR and loop-mediated isothermal amplification (LAMP) to this pressing public health issue. Equally important, by obtaining *Salmonella* counts by the best available culture-based technique in parallel, this project will offer reliable counts of *Salmonella* in Turkish biosolids in a manner that allows comparison to international numbers.

A pressing issue regarding the application of molecular based methods for counting pathogens in biosolids is that non-viable DNA may be amplified by these techniques, which would in turn lead to over-estimation of cell counts. A recently developed and verified technique for distinguishing the genetic material in intact, viable cells from genetic material outside of cells or contained in mortally damaged cells has been developed and demonstrated. This technique, which is based on the binding of the chemical propidium monoazide (PMA) to extracellular DNA only, potentially offers the most rapid and reliable method for distinguishing viable and non-viable cells to date, thus potentially solving the final challenge that faces molecular *Salmonella* quantification. Recently digested biosolids will be collected from a handful of municipal sewage treatment works in Turkey. *Salmonella* in samples were analyzed in parallel by three methods: 1) USA EPA method 1682 for determination of most probable number by culture-based techniques, 2) quantitative PCR (qPCR) with application of propidium monoazide and Taqman probes and 3) loop-mediated DNA amplification with PMA treatment. Initial results of these studies will be presented.

Keywords: *Salmonella*, loop-mediated isothermal amplification, qPCR, propidium monoazide, biosolid

1. Salmonella

Salmonella is a gram-negative bacterium capable of facultative intracellular anaerobic growth in animal cells, causing a spectrum of diseases generally referred to as salmonellosis. While medical and public health progress over the last 100 years have yielded remarkable improvements in the incidence of typhoidal (systemic) salmonellosis [1], the other main type of salmonellosis, enterocolitis or diarrhea, remains a very important disease world-wide and does not show signs of decreasing [2, 3].

The most direct and reliable measure of impact, deaths, show approximately 2100 deaths per year throughout Eastern Europe (including Turkey) [4] and 600 in USA per year [5]. A recent estimation of the total global burden, taking into account under-reporting rates, estimated 94 million cases and

155,000 deaths worldwide [4], a burden that falls disproportionately upon children younger than, making it one of the more significant global public health threats.

Despite massive global progress in sanitation, diagnosis, epidemiology, and treatment of infectious diseases, non-typhoidal salmonellosis does not show overall signs of decreasing, even in the developing world [3], likely due to trends such as mass food production and global transport [6]. Certain factors unique to *Salmonella* play important roles in this trend also. It is a durable organism, capable of non-pathogenic lifestyle, and highly virulent. Particularly interesting to the *Salmonella*, it is a zoonosis, a pathogen capable of causing disease in a range of animals; most serotypes can also infect certain animals that are used for food, such as chickens, turkey, and pigs [7]. Animal breeding operations can thus serve as reservoirs and transmission routes for infection.

In very general terms, treatment of municipal wastes yields two separate products; water and solid. The solids phase is generally referred to as "biosolid" [8]. As an agricultural amendment, biosolids offer many agricultural advantages such as nutrients (nitrogen and phosphorous) and bulk organic matter [8]. Biosolids are thus a valuable resource whose safe, pathogen-free exploitation offers many advantages in an increasingly populated and hungry world.

During waste treatment processes, microbes tend to be concentrated in the solid phase as opposed to the liquid phase, making biosolids the focus of concern in regards to pathogens [9]. The USA Class A regulations reflect the durability and virulence of *Salmonella*; while fecal coliform can be no higher than 2 million colony forming units (CFU) per 4 g, no more than 3 most probable number (MPN) per 4g *Salmonella* is allowed. The existing regulations regarding *Salmonella* are two decades old at this point and have been called into question regarding their safety for a number of reasons [10]. The "less than 3 MPN per 4 g limit" for *Salmonella* depends on certain very crucial assumptions; 1) that measurement techniques are accurate and 2) the measured number actually reflects the number of viable organisms present when the biosolid is applied. These two assumptions are currently points of uncertainty in the field.

A systematic analysis of *Salmonella* numbers mesophilically digested biosolids in 18 cities across the USA found counts ranging from below detection limit to 13.4, with a median value of 6.1 [11]. In a long-term monitoring study of a single plant in Arizona, *Salmonella* counts were found to be quite stable over a period of 18 years between 20 and 45 [11]. Similar numbers were reported by Wong *et al*., who reported that mesophilic digestion reduced counts from 194 MPN/ 4g to below detection limit [12]. Given the wide range of time and location, the conclusion can be drawn that mesophilic digestion generally reduces the number of *Salmonella* as measured by traditional methods to between 0-40 approximately in urban wastewater treatment plants, although this does not take VNBC or counting methods into account.

Current data suggests that *Salmonella* are reaching surface waters and food supply chains in Turkey currently, although available data is somewhat sparse. Aytac *et al*. [13] demonstrated that a remarkable number of leafy green vegetables that were irrigated using stream water downstream of Ankara tested positive for the presence of *Salmonella*, suggesting that released urban wastewater or land-applied biosolids were a significant source. The frequency of detection was significantly higher than is typically found in developing countries; for example, while over 10% of Turkish samples were positive, less than 1% of specimens in Spain have been reported as contaminated in similar studies. Similarly, Altdug et. al [14] identified *Salmonella* in several species of marine molluscs in the Sea of Marmara and Kacar *et al*. detected *Salmonella* in the waters of the Aegean.

2. Quantification by culture-based techniques

Although culture-based MPN approaches have been demonstrated to be accurate, they are slow, taking several days and much labor and supplies [15]. Additionally, enrichment does not necessarily resuscitate viable but non-culturable (VNBC) bacteria, which can stay non-culturable even after 5 days incubation in enrichment media for 5 days at 35ºC before re-emerging at a later time [16]. The

EPA 1682 method published in 2006 "*modified semisolid Rappaort-Vassiliadis (MSRV) medium MPN technique*", is the current gold-standard for quantification of *Salmonella* in biosolids. It is a precise, well-researched technique aimed at accurate quantification for analysis of the safety of Class A biosolids and is also the accepted method for analysis of Class B biosolid quality [12]. The 1682 method is a maximum probable number (MPN) technique which employs several strategies for enrichment, selection, and biochemical confirmation in order to address the many acknowledged challenges in consistently isolating *Salmonella* from biosolids.

While the strategies of the 1682 method are complete and widely used, there are certain significant disadvantages such as the lingering VNBC questions; what proportion of *Salmonella* will not grow during the initial enrichment stage but are actually able to emerge under the correct conditions, possibly months later? The additional obvious disadvantage is the time, labor, and materials requirements. MPN counting employs a large number of culture tubes per sample and several stages of sequential culturing requiring up to a week. A reliable molecular approach by molecular methods would require only one day of labor ideally and permit the analysis of a large number of samples simultaneously, drastically reducing labor and time.

2.1. Injury and the viable but non-culturable state

The relevance of viable but non-culturable (VNBC) state in regards to pathogens remains a subject of debate. While some pathogens such as *Escherichia* and *Salmonella* do have the ability to live outside of their host, they face hostile environmental conditions such as starvation, non-ideal temperatures or salinity, competition, and predation [17]. Such a situation might favor a dormant VNBC state from which cells might only emerge under certain conditions or signals consistent with entry to a host [17]. There have been a handful of well-designed studies which appear to present strong evidence for resuscitation of gram-negative, non-sporulating VNBC bacteria involving *Micrococcus luteus* [18], *Vibrio* [19] and *Campylobacter jejuni* [20]. Additionally, it has been demonstrated that *Salmonella* may produce and respond to a resuscitation promoting factor in the exit from a dormant state [21] presenting strong evidence that VNBC is indeed a real phenomenon in *Salmonella*.

3. Quantification of *Salmonella* by pcr-based techniques

The many challenges associated with culture-based techniques for quantification of pathogenic bacteria in biosolids and other media of public health concern have motivated a shift to an entirely different detection strategy. By measuring biomolecules specific to the pathogen of interest, problems such as competing organisms and VNBC-state cells can be avoided altogether. Amongst the available techniques for detecting genetic material with high specificity, polymerase chain reaction (PCR) based techniques are especially attractive due to the added benefit of exponential amplification of a signal, allowing for theoretical detection of single molecules of the target molecule. In practice however, the detection of single specific genetic markers is also faced with many technical challenges. The core technique, quantitative polymerase chain reaction (qPCR) for example is hindered by non-specific amplification, sensitivity, reaction inhibition by complex media (such as a biosolid), and detection of extracellular or genetic material in dead cells [22, 23]. Each of these issues is a topic of constant innovation.

3.1. Non-specific amplification

Non-specific amplification is primarily addressed in the selection of which target will be used as the indicator sequence; in the case of qPCR, design of primers and/or probe sequences. The design and validation of a primer/probe system is a lengthy process; in the case of *Salmonella*, a particular primer set for the metabolic gene *invA* [24] is well-accepted as being both unique to *Salmonella,* as in it does not yield significant signal from other bacteria, while also being common to known *Salmonella* strains.

Application of a Taqman system has been routinely demonstrated to reduce detection limits in complex media from thousands of targets down to 1-10. In the case of *Salmonella*, this increased specificity is an absolute necessity: Properly applied mesophilic digestion typically reduces *Salmonella* numbers down to the range of 1-200 cells per 4 g dry weight [25], meaning that a generic qPCR approach will more often than not fail to detect anything at all, despite the fact that these cell concentrations are of public health concern. An *invA* Taqman probe system has been successfully applied in a number of studies to date aimed at quantifying *Salmonella*, achieving detection sensitivities down to 5.8 copies per qPCR reaction [26-29]

3.2. Differentiation of live and dead genetic material

Mesophilic sludge treatment processes, currently the most popular pathogen reduction process in the developed world, are expected to reduce the viable number of pathogens by several orders of magnitude for example; however, how many intact DNA sequences are still present extracellularly or in dead cells (with ruptured membranes for example) is not well-understood [30], thus any qPCR-based quantification that neglects to address this issue can be called into question. Recently, an entirely new approach that avoids this pitfall has been under development however. The chemical propidium monoazide (PMA) has been validated over the last few years as being able to make this differentiation at more than a 99% rate; PMA is mixed with the sample of interest and exposed to light for 10-20 minutes, during which it forms covalent bonds only with the DNA that it encounters. PMA does not penetrate intact cells at an appreciable rate, thus DNA in viable cells is unaffected. Extracellular DNA is chemically modified in such a way that it can no longer serve as template for PCR, the remaining PMA is destroyed by the light treatment, and DNA that is inside of viable cells can then be safely extracted by the DNA extraction procedure. Nocker *et al*. recently demonstrated that the PMA technique is highly effective for *Salmonella* in complex environmental samples [31]. However, *Salmonella* in biosolids have not yet been subjected to this approach.

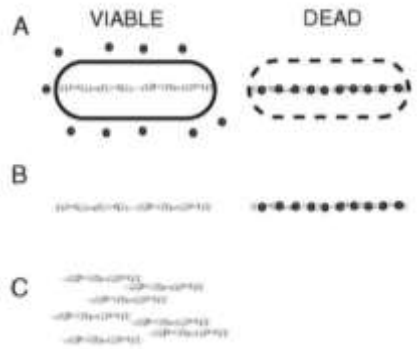

Figure 1. Representation of the photo-crosslinking process. At stage A, PMA (black circles) cannot access the DNA in cells with intact membranes while forming cross-links in all other DNA. After extraction and cleanup, these crosslinks in “dead” DNA remain. During PCR, only DNA from viable cells can be amplified (C). From [32]

4. Application of loop-mediated amplification to salmonella

Loop-mediated isothermal amplification (LAMP) is a relatively new technique in molecular biology that offers a rapid, inexpensive, resistant to inhibition, and highly-specific alternative to classical polymerase chain reaction (PCR). Techniques based on classical thermocycled PCR are currently among the primary methods for analysis of pathogens in environmental media. One of the main advantages of LAMP is that the amplification is isothermal, thus requiring only a single inexpensive heat block or water bath. Further, the high-specificity of LAMP greatly reduces the likelihood of false-positives, which PCR is very prone to. The polymerization enzyme used in LAMP is also highly resistant to conditions that would inhibit *Taq* polymerase, the classical PCR DNA polymerase

enzyme. Due to these advantages, LAMP is increasingly being explored as a superior method for the detection and quantification of specific pathogens in food and water supplies [33]. In regards to detection and copy number estimation, classical PCR offers no advantages over this newer LAMP technique.

Table 1. Comparison of the major properties, advantages, and disadvantages of LAMP and thermocycled PCR

Property	LAMP	Thermocycled PCR
Reaction time	30 min. – 1 hr.	2-3 hrs.
Specificity	High	Low
Resistance to inhibitors	High	Low
Clonable product	No	Yes
Detection	Naked eye or spectrophotometer	Gel electrophoresis
Temperature	~60°C constant	30-40 cycles of ~55°C-72°C-95°C
Primers	4-6	2

5. Current research

Recently digested biosolids will be collected from a handful of municipal sewage treatment works in Turkey. *Salmonella* in samples will be analyzed in parallel by three methods: 1) USA EPA method 1682 for determination of most probable number by culture-based techniques 2) qPCR with application of propidium monoazide (PMA) for elimination of extra-cellular DNA and 3) LAMP with PMA. Results of initial analyses of four municipal sewage treatment plants in Turkey will be presented.

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