Multi-laboratory evaluations of the performance of *Catellicoccus marimammalium* PCR assays developed to target gull fecal sources

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Abstract

This manuscript reports results from a multi-laboratory (n = 11) evaluation of four different PCR methods targeting the 16S rRNA gene of *Catellicoccus marimammalium* originally developed to detect fecal contamination from gulls in coastal environments. The methods included conventional end-point PCR, a SYBR® Green qPCR method, and two TaqMan® qPCR methods. Different techniques for data normalization and analysis were also tested. Data analysis methods had a pronounced impact on assay sensitivity and specificity calculations. Across-laboratory standardization of metrics including the lower limit of quantification (LLOQ), target detected but not quantifiable (DNQ), and target not detected (ND) significantly improved results compared to results submitted by individual laboratories prior to definition standardization. The unit of measure used for data normalization also had a pronounced effect on assay performance. Data normalization to DNA mass improved quantitative method performance as compared to *enterococcus* normalization. The MST methods tested here were originally designed for gulls, but they also detected feces from other birds, particularly feces from pigeons found at the coast. Some pigeon feces from California were found to contain sequences similar to *C. marimammalium* from gull feces. However, the prevalence, geographic scope, and ecology of *C. marimammalium* in host birds other than gulls are unclear and still require further investigation. This study represents an important first step in the multi-laboratory assessment of these methods and highlights the need to broaden and standardize additional evaluations, including environmentally relevant target concentrations in ambient waters from diverse geographic regions.

Introduction

To prevent potential exposure to recreational waters contaminated with sewage or other sources of human fecal material, concentrations of fecal indicator bacteria (FIB) such as enterococci and *E. coli* are used to monitor microbial water quality. Fecal indicator bacteria also are found in animal feces and therefore animals are considered potential sources of fecal pollution. Of particular importance to pollution of coastal waters are waterfowl, as their fecal droppings can be found in significant numbers at the shoreline, and their feces can carry FIB (Alderisio and DeLuca 1999, Grant *et al.* 2001, Haack *et al.* 2003, Wright *et al.* 2009), human pathogens (Quessy and Messier 1992, Graczyk *et al.* 1998, Lévesque *et al.* 2000, Albarnaz *et al.* 2007, Bart *et al.* 2008, Kinzelman *et al.* 2008, Lu *et al.* 2011), and virulence genes (Radhouani *et al.* 2011, Poirel *et al.* 2012). There is evidence that waterfowl fecal sources might carry different human health risks than human fecal sources such as sewage (Schoen and Ashbolt 2010, Soller *et al.* 2010). Reduced risk may arise in part because bird feces can contain novel species within a pathogenic genus, most of which may not present a significant human health risk (Lu *et al.* 2011). However, a significant number of beach closures could be attributed to waterfowl as they are recognized as FIB sources to coastal waters and inland recreational waters (Standridge *et al.* 1979, Lévesque *et al.* 2000). Hence, there is a need to identify when waterfowl are the primary fecal pollution sources from both risk assessment and local economy standpoints.

Microbial source tracking (MST) is a tool to help identify fecal sources impacting microbial water quality, to help devise effective remediation strategies, and to more accurately determine health risk of different pollution sources (Santo Domingo *et al.* 2007). Gulls are especially significant contributors to bird fecal contamination of beaches and coastal waters, particularly at urban recreational beaches. There have been several MST assays developed with the intent to specifically measure gull fecal contamination in environmental samples, and most of these assays target the 16S rRNA gene of *Catellicoccus marimammalium*. Interestingly, other MST waterfowl assays have been developed, several based on *Catellicoccus*-like 16S rRNA gene sequences (Green *et al.* 2012, Ryu *et al.* 2012), suggesting that members of this genus and other closely related bacterial species are widespread in waterfowl.

Relevant to this study, the Gull2 marker has been commonly found in gull feces with limited cross-reactivity to other animal feces, with the exception of a few seabird species such as pelican (Lu *et al.* 2009, Ryu *et al.* 2012). The Gull2 marker was originally used as part of a presence/absence assay and when coupled with SYBR Green chemistry it became possible to use it as a quantitative assay. The Gull2SYBR assay detected gull feces with a relatively low detection limit (i.e., 0.0006 ng of gull fecal DNA per PCR reaction) and the marker has been detected in waters with a known history of gull fecal pollution (Lu *et al.* 2011). The occurrence of

this marker also positively correlated to the amount of *Campylobacter* spp. in gull feces (Lu *et al.* 2011), suggesting its potential value at predicting human health risks. This Gull2 *C. marimammalium* marker appears to be widely prevalent in gulls from a variety of geographic regions, and this marker has been found in gulls from both North America and Europe (Ryu *et al*. 2012).

One of the limitations of SYBR Green qPCR assays is the difficulty of quantifying the targeted genetic sequence when spurious amplification occurs. To circumvent this problem, the Gull2 assay was successfully modified as a TaqMan-based assay by developing a 5′-exonuclease fluorogenic probe to use in conjunction with the original Gull2 primers (Sinigalliano *et al*. 2010). This Gull2Taqman assay was used to test waters at a subtropical beach during an epidemiological study (Sinigalliano *et al.* 2010, Shibata *et al.* 2010). The occurrence and signal intensity of the Gull2Taqman assay positively correlated to independent camera-system measurement of gull abundance at beaches (Solo-Gabriele *et al.* 2011). In addition, this study reports an evaluation of a new qPCR assay for detection of gull feces, the LeeSeaGull assay (Lee *et al.* 2013). This LeeSeaGull assay is based on detection of the same target region of *C. marimammalium* as the Gull2 assay, but has different primer/probe sequences and amplifies a smaller PCR product. Several of the available bird MST assays target a similar region of the 16S rRNA gene of *C. marimammalium*. An alignment of the primer and probes target sites of these various assays in relation to a reference *C. marimammalium* sequence is show in Figure SI-1.

While reports suggest that these assays targeting *C. marimammalium* are useful in studies to detect bird fecal contamination, there has been limited cross-laboratory evaluation. Such studies are needed to address critical issues such as host-specificity, detection limits, host-distribution, and marker relative abundance in targeted and non-targeted hosts. Overall, method evaluation and standardization are important for implementation of MST technologies into environmental monitoring programs.

The work reported here was conducted by 11 different participating laboratories. This study examined the performance of several gull assays by challenging them with a set of purified DNA samples extracted from single-source and mixed fecal slurries from different animals, sewage, and septage (Boehm *et al.* 2013). The study highlights the findings of this effort and discusses some limitations observed with regard to data analysis, including effects on assay specificity and sensitivity. This effort was part of a broader multi-laboratory assessment of MST method performance, the Source Identification Protocol Project (SIPP). Other host targets and aspects of assay performance are reported elsewhere (e.g., Boehm *et al.* 2013, Layton *et al.* 2013, Schriewer *et al.* 2013, Ebentier *et al.* 2013, Raith *et al.* 2013, Stewart *et al.* 2013, Ervin *et al.* 2013).

Methods

Preparation and Processing of Challenge Samples

The collection of fecal material and preparation of replicate challenge sample filters for multi-laboratory comparative analysis has been described in detail elsewhere (Boehm *et al.* 2013). Briefly, challenge samples were created from freshly collected fecal material from 12 different positively identified sources: individual humans, sewage, septage, horses, cattle, deer, pigs, geese, chickens, pigeons, sea gulls, and dogs. Two independent fecal composites were generated from multiple individuals for each fecal host source. For example, in the case of pigeons, pigeon fecal samples were collected from four regional locations along California. Samples were taken from coastal, recreational, and residential areas, all variable distances from the coast. Tarps were set out and pigeons were attracted in order to collect fresh feces. Only droppings that were visually observed to originate from pigeons were collected. Each collection consisted of multiple individuals (i.e., >20/region) in order to create each of the two pigeon fecal composites. From the combination of all the various host-source fecal types, thirty-two total types of challenge samples (Table SI-1) were created from fecal slurries and/or sewage or septage in either "singleton" (individual fecal source) or "doubleton" mixtures (two fecal sources in 90%:10% ratios by volume). Fifty ml of the blinded, composite slurries were filtered for distribution. Some of the challenge samples included singletons at 1:10 strength to assess method sensitivity (these were created by filtering only 5 ml of slurries), thus the 1:10 samples were identical to the "full-strength" samples, just with $1/10^{th}$ of the fecal slurry by volume. The filtered samples were then shipped in duplicate (n = 64) to participating laboratories on dry ice. More specific details about the sample collection, compositing, processing, and distribution to laboratories can be

found in the SI and also in the SIPP study overview paper (Boehm *et al.* 2013).

A total of eleven laboratories participated in gull methods assessment: six laboratories tested the Gull2Taqman assay, four laboratories tested the Gull2SYBR assay, and four laboratories tested the Gull2Endpoint assay (Supplemental Information (SI) Table SI-1; ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_523_539SI.pdf). In addition, three laboratories tested the LeeSeaGull assay (Table SI-1). Data from one of the laboratories was presented in the overview paper (Boehm *et al.* 2013). Data reported here from the other two laboratories represents additional analysis that is not represented elsewhere. Quality control guidelines were promulgated to the participating labs, and QC filter blanks and sample controls were handled by the lab originating the samples, while extraction and molecular controls were handled by each participating lab. Details on the results for filter blanks and other controls analyzed at the core labs are discussed in the overview paper (Boehm *et al.* 2013). QC of all processing controls (extraction controls, no template PCR controls, etc.) was left up to the individual laboratories to check before submitting their data.

Filter Processing and DNA Extraction

Individual laboratories extracted and purified total genomic DNA from the frozen filters, typically with some variant of bead beating lysis and DNA purification with commercially available kits (see Table SI-3 for details). The additional evaluation of the LeeSeaGull assay utilized DNA from each of the 64 samples. To obtain sufficient volumes for this additional analysis with the LeeSeaGull assay, DNA was pooled from three of the participating laboratories, duplicate aliquots were created, and these were supplied to two additional Laboratories (5 and 6) for the additional analysis.

PCR and MST Target Analysis of *C. marimammalium*

Primer and probe oligonucleotide sequences for the tested assays are shown in Table SI-4, and their hybridization target locations are shown in a sequence alignment relative to a reference *C. marimammalium* 16S rDNA gene sequence in Figure SI-1. Protocols used in different laboratories for each assay were similar but variations existed between some laboratories with regard to reagents, cycling platforms, and in some cases cycling conditions, as well as different probe quencher chemistries. Briefly, protocols for the Gull2SYBR qPCR and Gull2Endpoint PCR assays were based on Lu *et al.* (2009), the Gull2Taqman qPCR assay based on Sinigalliano *et al.* (2010), and the LeeSeaGull assay based on Lee *et al.* (2013). Further information is provided in the SI, with protocol variations by laboratory detailed in Table SI-5.

Sequencing Verification of PCR Amplicon Identity from Pigeon Fecal Samples

Due to the consistent high level of amplification seen with these particular pigeon fecal samples across laboratories for all of the *Catellicoccus*-based qPCR assays, a representative amplicon sample of each of the two SIPP Study pigeon composite samples from both the Gull2Taqman and the LeeSeaGull assay were sequenced with the Gull2 and LeeSeaGull assay primers respectively. The qPCR products from the composite pigeon feces samples were purified with commercial PCR purification kits, and sequenced using an ABI Prism 3730 DNA Analyzer with both forward and reverse primers. This pigeon sequence data was combined with available *Catellicoccus* sequence data from a variety of other shorebirds. This is presented in Figure 1 as a multiple-sequence alignment of the primers and probes from these two gull assays with the *C. marimammalium* reference sequence and with *Catellicoccus* and *Catellicoccous*-like consensus sequences from a variety of shorebirds, including gulls, pigeons, cranes, snow geese, Ruddy Turnstones, Red Knot Sandpiper, and Semi-palmated Sandpiper. The sequences generated from these other shorebirds are provided by the laboratory of Jorge Santo Domingo and are part of another ongoing study (Grond *et al.* unpublished data). More details on the sequencing can be found in the SI.

Processing and Analysis of Multi-Laboratory Molecular Data

Data analysis was standardized to better compare data sets between different laboratories performing the same quantitative assay (see SI for details). Briefly, pooled master standard curves were created for each laboratory including the lowest concentration standard in which amplification was detected in at least 80% of replicates. Outliers were removed based on regression curve standardized residual values of >+3 or <-3. The lower limit of quantification

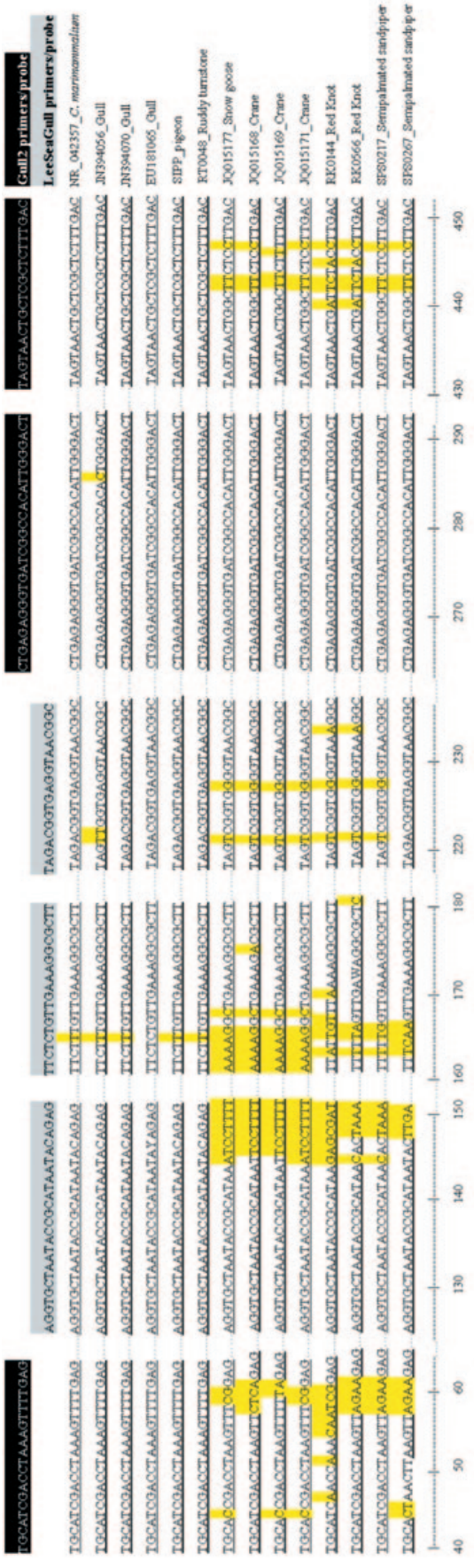

Figure 1. Comparison of *Catellicoccus marimammalium* and *Catellicoccus*–like clone sequences from different waterfowl and shore birds. Sequences highlighted in black and gray represent sequences for the Gull2-Taqman assay and LeeSeaGull assay primers and probes, respectively. Sequences from the gulls, cranes and shorebirds (Red Knot and Semi-palmated Sandpiper) represent consensus sequences from multiple clone libraries generated from DNA extracted from individual fecal samples. Sequences from shorebirds are part of an ongoing study (Grond *et al.* unpublished data). Yellow highlighting indicates differences in individual gene sequences and consensus sequence.

(LLOQ) was calculated from these standard curves as the average C_t value of the lowest concentration in the standard curve. Resulting standard curve statistics are shown in Table 1. For sample analysis, some laboratories ran duplicates and some triplicates; therefore, a strategy was devised to standardize interpretation of results across laboratories with regard to being within the range of quantification (ROQ), detected but not quantifiable (DNQ), or not detected (ND; see SI).

Quantitative results both before and after data analysis standardization were normalized by abundance of viable enterococci (membrane filtration), total Bacteroidales (genbac3 qPCR assay; Siefring *et al.* 2008), and by mass of DNA, all as measured in the original sources. These measurements were provided to the participating laboratories (Boehm *et al.* 2013). The geometric means of the measurements made at multiple laboratories were used for data normalization by total Bacteroidales and by mass of DNA. Sensitivity and specificity were calculated for different analysis scenarios using the criteria as presented in Boehm *et al.* (2013), before and after standardization of data processing, and under the defined analysis parameters of treating pigeon as a true positive and treating DNQ results as true negatives.

Results

On average, all four assays tested in this study detected gull feces at "high" sensitivity (>80% as defined by Boehm *et al.* 2013), although differences between laboratories in sensitivity and specificity performance were noted. Standardized data analysis (Table 1) generally increased assay performance, particularly for the Gull2Taqman and LeeSeaGull qPCR assays (Table 2). Average across laboratory %sensitivity/%specificity was 92/96 for Gull2Taqman, 100/86 for LeeSeaGull, 88/89 for Gull2Endpt, and 73/96 for Gull2SYBR, under the following defined analysis conditions: standardized data analysis, pigeons considered a true positive, and DNQ a true negative (Table 2).

Apparent performance varied depending upon how data were normalized (Figures 2

Table 1. Standard curve statistics for gull assays with standardized post-processing.

Assay	Lab	Slope	Y-Intercept	R^2	Efficiency (%)	LLOQ (C_t)	LLOQ (cp/rxn)
Gull2Taqman	4	-4.88	58.6	0.99	60.4	41.8	2794
	5	-3.7	41.7	0.98	86.5	36.2	31
	6	-3.43	41.2	1	95.6	36.7	20
	7	-3.66	40.8	0.99	87.7	35.8	23
	8	-3.79	42.2	0.98	83.7	36.6	31
	9	-3.4	38.6	0.99	97	34.5	16
Gull2SYBR	1	-3.66	38.3	0.99	87.4	35.6	5
	2	-3.54	36.4	0.99	91.7	32.3	15
	3	-3.23	31.2	0.93	104	27.9	11
	4	-3.72	42.3	0.99	85.6	33.3	249
LeeSeaGull	5	-3.43	45.6	1	95.5	38.8	97
	6	-3.42	44.1	1	95.9	36.4	180
	10	-4.26	50	0.99	71.7	36.6	1402

Table 2. %Sensitivity (sens) and %specificity (spec) for each assay with varying sets of defined analysis parameters. Results are based on all 64 challenge samples without normalization to enterococci, Bacteroidales, or DNA mass. Results are presented by individual laboratory and as an average of all laboratories performing the assay. For the quantitative assays (Gull2Taqman, Gull2SYBR, LeeSeaGull), calculations were performed with three sets of defined analysis parameters: with the original submitted data set, with standardized data processing, and with standardization and treating pigeon as a true positive and DNQ as a true negative. For the conventional PCR assay (Gull2Endpoint), calculations were performed on two versions of the data: the original submitted data set, and after including pigeon as a true positive. Additional analysis can be found in the Supplemental Information.

	Lab 4		Lab 5		Lab 6		Lab 7		Lab 8		Lab 9		Average	
Gull2Taqman	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**
Original submitted results	58.3	94.2	91.7	11.5	100.0	92.3	100.0	67.3	100.0	67.3	100.0	69.2	91.7	67.0
Standardized analysis	66.7	92.2	91.7	9.6	100.0	92.3	100.0	80.8	100.0	80.8	100.0	82.7	93.1	73.1
Standardized analysis, pigeon +, DNQ-	57.1	98.0	92.9	78.0	100.0	100.0	100.0	100.0	100.0	98.0	100.0	100.0	91.7	95.7

	Lab 1		Lab 2		Lab 3		Lab 4		Average	
Gull2SYBR	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**
Original submitted results	83.3	96.2	100.0	96.2	91.7	80.8	83.3	90.4	89.6	90.9
Standardized analysis	50.0	96.2	100.0	96.2	91.7	76.9	83.3	90.4	81.3	89.9
Standardized analysis, pigeon +, DNQ-	50.0	100.0	100.0	100.0	78.6	84.0	64.3	98.0	73.2	95.5

	Lab 5		Lab 6		Lab10		Average	
LeeSeaGull	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**
Original submitted results	100.0	7.7	100.0	9.6	100.0	94.2	100.0	37.2
Standardized analysis	100.0	40.4	100.0	32.7	100.0	75.0	100.0	49.4
Standardized analysis, pigeon +, DNQ-	100.0	68.0	100.0	90.0	100.0	100.0	100.0	86.0

	Lab 4		Lab 7		Lab 8		Lab 11		Average	
Gull2Endpoint	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**	**Sens**	**Spec**
Original submitted results	58.3	92.3	100.0	58.8	100.0	94.2	83.3	96.2	85.4	85.4
Pigeon +	64.3	96.0	100.0	61.2	100.0	98.0	85.7	100.0	87.5	88.8

through 7). Pigeon samples amplified in all six laboratories at concentrations slightly higher than gull samples. Most samples that cross-reacted with other non-gull sources were from a single laboratory, and no cross-reactivity was consistent across all laboratories. Most cross-reactivity was observed at low target concentrations (i.e., at high C_t values), with good separation between target and non-target samples (Figures 2 through 7). These figures may be a bit misleading as to the true extent of the DNQ prevalence, since the symbols can overlap each other. Therefore, Tables 3, 4, and 5 present the total number and percentage of challenge samples that were not detected, detected but not quantifiable, or within the range of quantitation for the Gull2Taqman, Gull2SYBR, and LeeSeaGull qPCR assays.

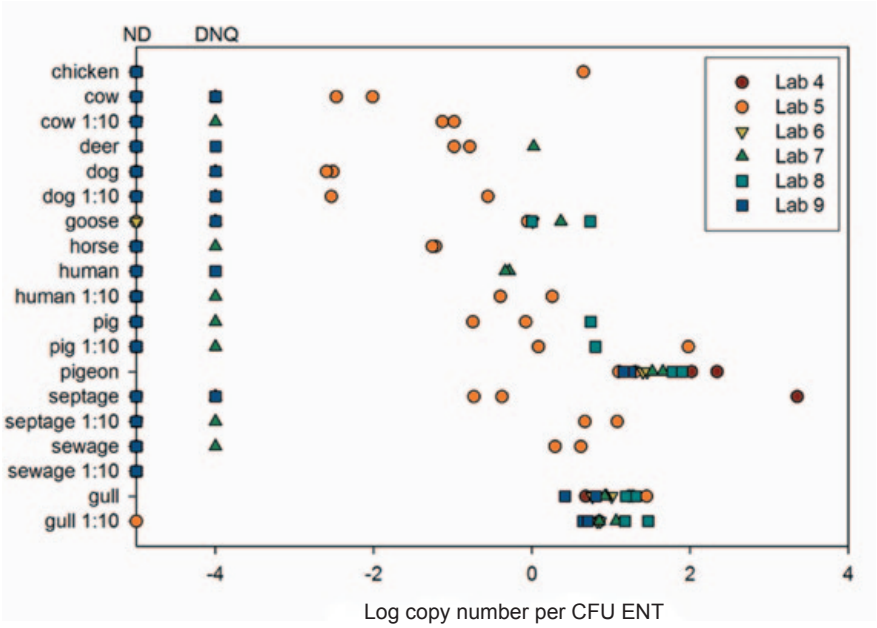

Figure 2. Original submitted non-standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by viable enterococci CFU measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

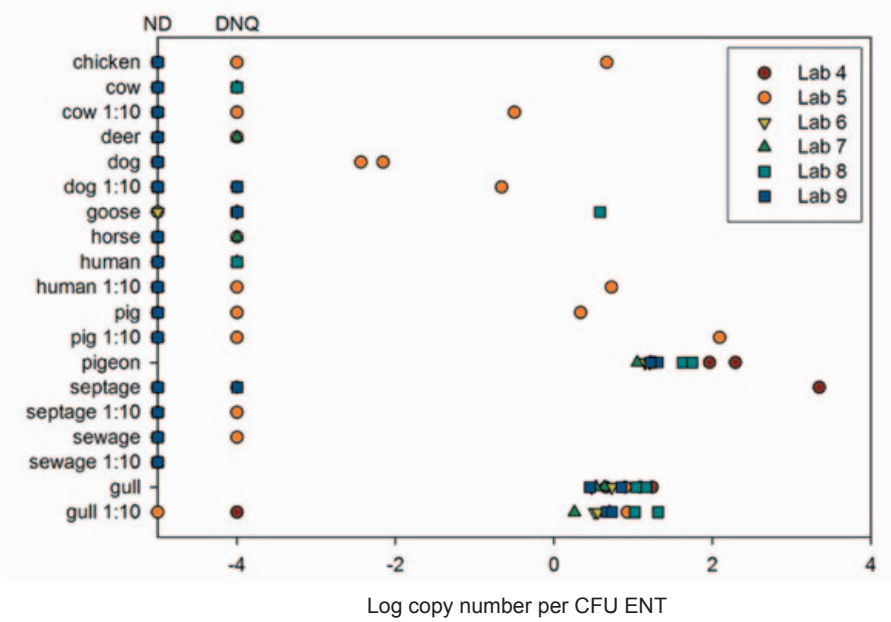

Figure 3. Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by viable enterococci CFU measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

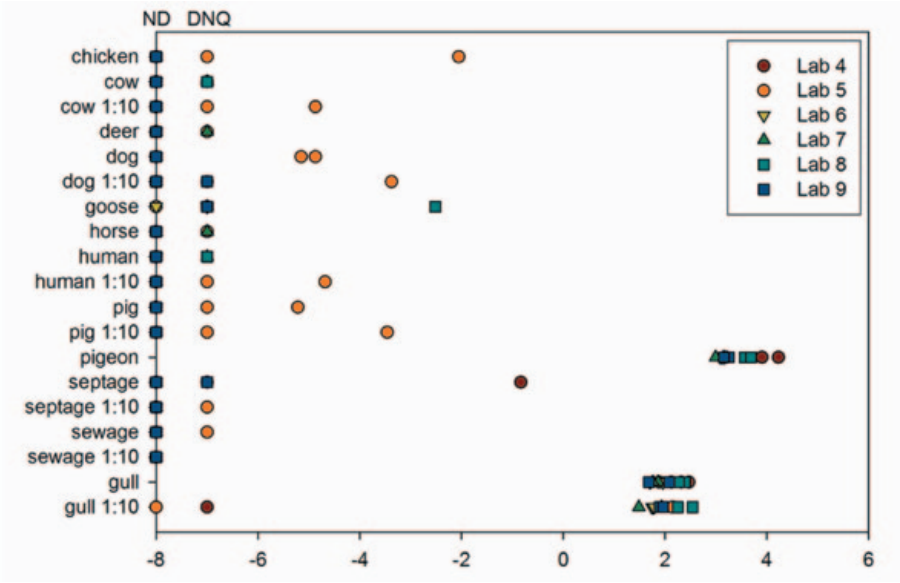

Figure 4. Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by Bacteroidales genbac3 copy number measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

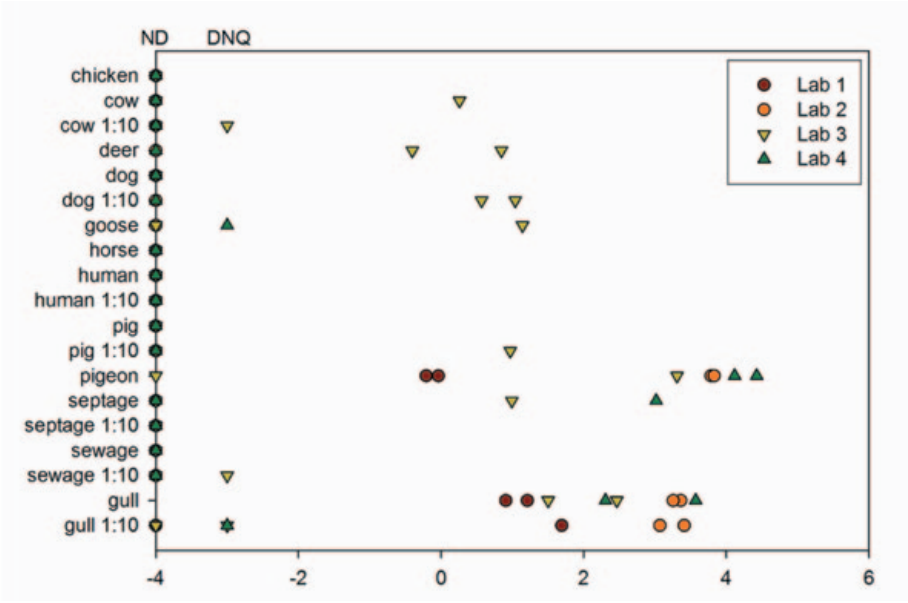

Figure 5. Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

Gull2Taqman Assay

The non-standardized data as submitted by each of the six laboratories performing the Gull2Taqman assay are given in Figure 2, whereas Figure 3 shows the same plot using standardized data analysis. Standardization of data analysis removed much of the cross-reactivity observed (Figures 2 and 3). Most non-target samples that were reported within the quantifiable range became DNQ, and many samples that were previously DNQ were labeled as not detected (ND). Shifts from DNQ to ND were due to amplification seen after 40 cycles (which was classified as ND under data standardization), or in cases where only 1 of 3 replicates amplified (see SI). Standardization of data analysis removed apparent cross-reactivity that was of a similar magnitude to gull samples in some cases (Laboratory 8 pig, Laboratory 5 septage, Laboratory 5 sewage).

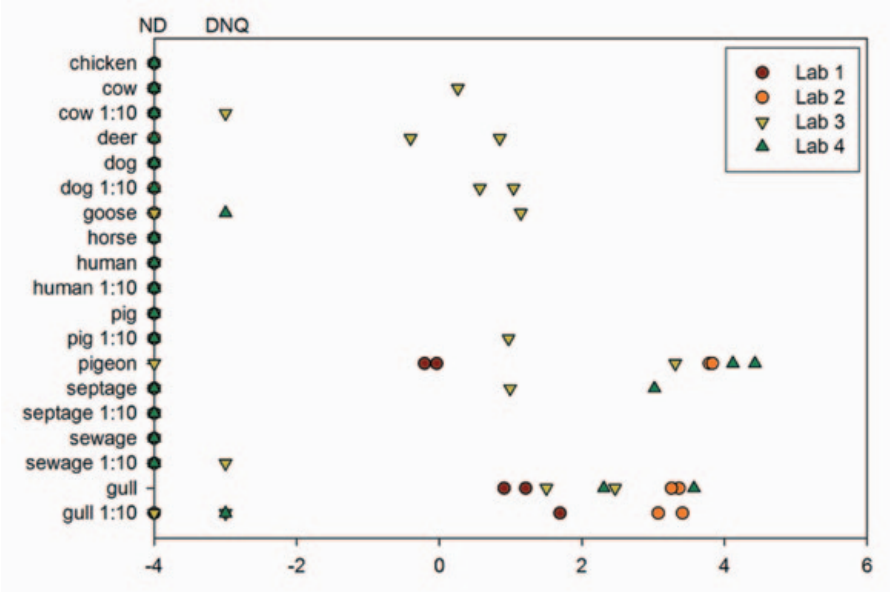

Figure 6. Standardized Gull2SYBR assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

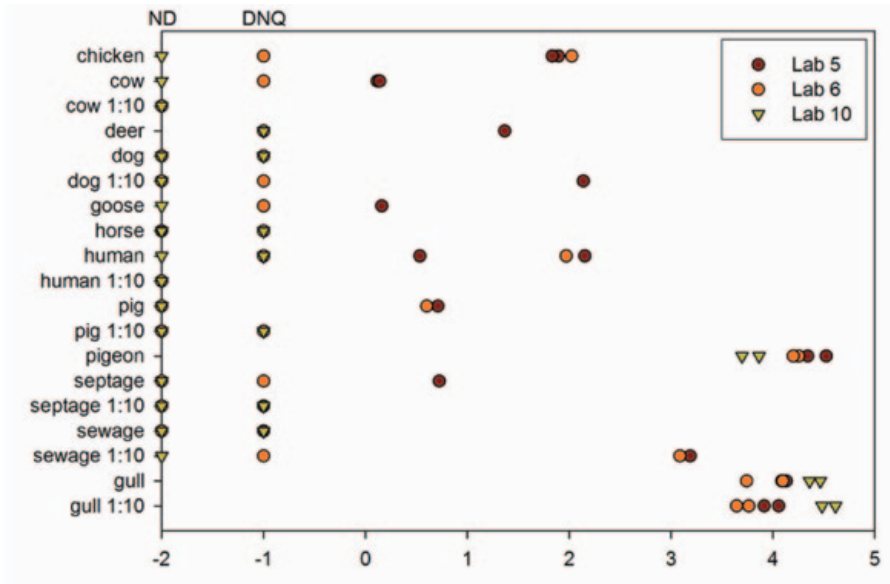

Figure 7. Standardized LeeSeaGull assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

Pigeon was a noteworthy exception to data standardization, in which case samples remained solidly positive (at concentrations comparable to that seen for gull feces).

Normalizing the data to Bacteroidales resulted in higher concentrations of *C. marimammalium* reported for the gull and pigeon samples, while the remaining non-target samples shifted to lower concentrations (Figure 4). This normalization effectively removed the overlap of non-target amplification with the gull samples (except for pigeon) that was observed in the plots based on enterococci normalization (Figures 2 and 3). There was greater than two orders of magnitude difference between the gull and pigeon samples and the nearest other non-target sample (Laboratory 4 septage). Pigeon samples again were seen at higher concentrations than gull samples when normalizing to Bacteroidales for the Gull2Taqman assay.

Table 3. The number (and percent in parentheses) of challenge samples that were not detected (ND), detected but not quantifiable (DNQ), or in the range of quantification (ROQ) for the Gull2Taqman assay using standardized results from six labs (n = 12 for each source).

Source	GullTaqman		
	ND	DNQ	ROQ
Chicken	10 (83)	1 (8)	1 (8)
Cow	8 (67)	4 (33)	0 (0)
Cow 1:10	10 (83)	1 (8)	1 (8)
Deer	9 (75)	3 (25)	0 (0)
Dog	10 (83)	0 (0)	2 (17)
Dog 1:10	9 (75)	2 (17)	1 (8)
Goose	3 (25)	8 (67)	1 (8)
Horse	9 (75)	3 (25)	0 (0)
Human	10 (83)	2 (17)	0 (0)
Human 1:10	10 (83)	1 (8)	1 (8)
Pig	10 (83)	1 (8)	1 (8)
Pig 1:10	10 (83)	1 (8)	1 (8)
Pigeon	0 (0)	0 (0)	12 (100)
Septage	8 (67)	3 (25)	1 (8)
Septage 1:10	10 (83)	2 (17)	0 (0)
Sewage	10 (83)	2 (17)	0 (0)
Sewage 1:10	12 (100)	0 (0)	0 (0)
Gull	0 (0)	0 (0)	12 (100)
Gull 1:10	2 (17)	1 (8)	9 (75)

Table 4. The number (and percent in parentheses) of challenge samples that were not detected (ND), detected but not quantifiable (DNQ), or in the range of quantification (ROQ) for the Gull2SYBR assay using standardized results from four labs (n = 8 for each source).

Source	Gull2SYBR		
	ND	DNQ	ROQ
Chicken	8 (100)	0 (0)	0 (0)
Cow	7 (88)	0 (0)	1 (13)
Cow 1:10	7 (88)	1 (13)	0 (0)
Deer	6 (75)	0 (0)	2 (25)
Dog	8 (100)	0 (0)	0 (0)
Dog 1:10	6 (75)	0 (0)	2 (25)
Goose	5 (63)	2 (25)	1 (13)
Horse	8 (100)	0 (0)	0 (0)
Human	8 (100)	0 (0)	0 (0)
Human 1:10	8 (100)	0 (0)	0 (0)
Pig	8 (100)	0 (0)	0 (0)
Pig 1:10	7 (88)	0 (0)	1 (13)
Pigeon	1 (13)	0 (0)	7 (88)
Septage	6 (75)	0 (0)	2 (25)
Septage 1:10	8 (100)	0 (0)	0 (0)
Sewage	8 (100)	0 (0)	0 (0)
Sewage 1:10	7 (88)	1 (13)	0 (0)
Gull	0 (0)	0 (0)	8 (100)
Gull 1:10	2 (25)	3 (38)	3 (38)

Normalizing the data to DNA mass also shifted the data and increased the difference between target and non-target concentrations, but the shift was not as dramatic as with Bacteroidales normalization (Figure 5). For the DNA mass normalization, there was one non-target sample (other than pigeon) that overlapped the range of concentrations observed for gull samples (Laboratory 4 septage). Gull and pigeon concentrations showed more overlap when the data were normalized to DNA mass.

Gull2SYBR Assay

When data analysis was standardized for the Gull2SYBR assay and data were normalized to DNA mass in the single-source samples, signals for gull and pigeon samples ranged widely. Under these analysis conditions, the data ranged over several orders of magnitude across the four laboratories performing this assay (2+ log for gull, 4+ log for pigeon; Figure 6). Laboratory 1 concentrations for Gull2SYBR were much lower for gull and pigeon samples compared to the other three laboratories. Some diluted gull samples were classified as DNQ and ND (Laboratories 1, 3, and 4) and one pigeon sample was classified as ND (Laboratory 3). Other than pigeon, no non-gull samples consistently amplified within the quantifiable range across the four laboratories. Most of the non-target amplification was reported from a single laboratory (Laboratory 3). In addition, one of the replicate septage samples from Laboratory 4 showed cross-reactivity; this same septage sample also showed cross-reactivity with the Gull2Taqman assay.

LeeSeaGull Assay

Data analysis was standardized for the LeeSeaGull assay and data were normalized to DNA mass in the single-source samples (Figure 7). Two of the three laboratories used aliquots of the same pooled DNA. All gull and pigeon samples amplified within the quantifiable range, and at a similar concentration when normalized to DNA mass.

Table 5. The number (and percent in parentheses) of challenge samples that were not detected (ND), detected but not quantifiable (DNQ), or in the range of quantification (ROQ) for the LeeSeaGull assay using standardized results from three labs (n = 6 for each source).

Source	LeeSeaGull		
	ND	DNQ	ROQ
Chicken	2 (33)	1 (17)	3 (50)
Cow	2 (33)	2 (33)	2 (33)
Cow 1:10	6 (100)	0 (0)	0 (0)
Deer	0 (0)	5 (83)	1 (17)
Dog	4 (67)	2 (33)	0 (0)
Dog 1:10	4 (67)	1 (17)	3 (50)
Goose	2 (33)	3 (50)	3 (50)
Horse	2 (33)	4 (67)	0 (0)
Human	1 (17)	2 (33)	3 (50)
Human 1:10	6 (100)	0 (0)	0 (0)
Pig	4 (67)	0 (0)	2 (33)
Pig 1:10	3 (50)	3 (50)	0 (0)
Pigeon	0 (0)	0 (0)	6 (100)
Septage	4 (67)	1 (17)	1 (17)
Septage 1:10	4 (67)	2 (33)	0 (0)
Sewage	4 (67)	2 (33)	0 (0)
Sewage 1:10	2 (33)	2 (33)	2 (33)
Gull	0 (0)	0 (0)	6 (100)
Gull 1:10	0 (0)	0 (0)	6 (100)

Similarly to the other tested assays, most of the non-target amplification was reported from a single laboratory (Laboratory 5). Other than pigeon, none of the non-gull samples consistently amplified within the quantifiable range across the three participating laboratories. However, several of the samples that cross-reacted (chicken, human, and diluted sewage) did amplify at Laboratories 5 and 6, which used aliquots of the same DNA samples. The one human sample that cross-reacted was reanalyzed by laboratory 6 using a sample of original DNA (not the pooled sample) and resulted in a ND, suggesting that this particular pooled sample may have been contaminated. None of the concentrations for non-target samples were within the same range as for gull and pigeon samples when normalized to DNA mass for this assay. The closest cross-reactivity was observed in one of the sewage dilution replicates (Laboratories 5 and 6 diluted sewage) and was within one order of magnitude of the target samples. All other non-target samples were greater than one order of magnitude from target sample concentrations.

Gull2Endpoint Assay

The Gull2Endpoint assay data before standardization exhibited high sensitivity and specificity in two laboratories, high specificity (but not sensitivity) in one laboratory, and high sensitivity (but not specificity) in one laboratory (Table 2). Gull2Endpoint assay sensitivity and specificity from the four laboratories performing this assay improved slightly with the inclusion of pigeon samples as a true positive. Overall averages for sensitivity and specificity were good (>80%) regardless of pigeon classification. Within the four laboratories, sensitivity was 100% at Laboratories 7 and 8, but lower at Laboratories 4 and 11. Specificity was higher in Laboratories 4, 8, and 11 (>90%) than in Laboratory 7.

Sequencing of Amplicons from Gull2Taqman and LeeSeaGull Assays

Using the Gull2 primers, the sequence derived from pigeon fecal samples was identical to that of a reference *C. marimammalium* sequence (NCBI accession number: NR_042357). When using the LeeSeaGull primers, there was only a single base pair mis-match to this reference sequence out of a 112 bp amplicon. Thus pigeon fecal amplicons from both of these gull assays showed >99% sequence identity to a known *C. marimammalium* sequence. From Figure 1 it can also be seen that sequences from gull feces, pigeon, and ruddy turnstone are nearly identical to the *C. marimammalium* reference sequence (>99% identity). Thus in these species, the gull assays targeting *Catellicoccus* might be expected to produce positive signals. Indeed, besides seagull, *C. marimammalium* signals were detected in both pigeon and ruddy turnstone (Grond *et al.* unpublished data). While the *C. marimammalium* signals in the SIPP Study pigeon samples were relatively high, we were not able to determine the host distribution of this bacteria species in pigeons, as we only analyzed fecal composites rather than samples from individuals. In addition, 16S rRNA gene sequences have been identified from shorebirds, crane, and snow goose that are closely related to *Catellicoccus* spp., but which are significantly different to *C. marimammalium* (i.e., ≤95% identity). As there are several mismatches with the gull primers and probes, these avian species are presumably less likely to produce *C. marimammalium* signals.

Influence of Standardization of Metrics

For quantitative assays, percent sensitivity and specificity for individual laboratories and averages across laboratories were computed before and after standardized data processing (Table 2, standardized analysis). In addition, results were analyzed for standardized analysis under the defined analysis conditions of treating pigeon as a true positive and DNQ as a true negative (Table 2, standardized analysis, pigeon +, DNQ-). Additional sensitivity and specificity results are shown in Table SI-6, which consists of the original submitted results with the inclusion of pigeon as a true positive, the original results with pigeon as a true positive and DNQ as a true negative, and after standardized data processing while treating pigeon as a true positive. For the conventional PCR Gull2Endpoint assay, sensitivity and specificity was similarly calculated treating pigeon as a true positive (Table 2, pigeon +). The number of samples classified as ROQ, DNQ, and ND for each quantitative assay, for both the non-standardized original submitted results and after standardization data processing is shown in Table SI-7.

For the Gull2Taqman assay, the multi-lab averages for sensitivity and specificity were slightly improved by standardization of data analysis (Table 2). Marked improvement, >90% for both sensitivity and specificity, was observed with standardization of data analysis combined with pigeon considered as a true positive and DNQ as a true negative (Table 2). Sensitivities and specificities for individual laboratories also showed general improvement after data standardization and pigeon/DNQ reassignment. Only the sensitivity in Laboratory 4 and the specificity in Laboratory 5 remained below 90% (Table 2).

Specificity and sensitivity results for the Gull2SYBR assay were similar under the different analysis conditions (Table 2). Specificity was generally high under all analysis conditions (>90% in all laboratories except Laboratory 2), but sensitivity deceased with standardized data analysis with the average going below 80% when standardized analysis was combined with pigeon/DNQ reclassification. Laboratory 1 showed a decrease in sensitivity with data standardization, while Laboratories 3 and 4 showed a decrease with pigeon/DNQ reclassification.

Overall average specificity results for the LeeSeaGull assay showed some improvement with data standardization and a large improvement with standardized analysis combined with pigeon/DNQ reclassification. Sensitivity was 100% for this assay regardless of data analysis conditions. However, specificity varied widely even when using different data analysis approaches. Original data and standardized, pigeon/DNQ reclassified data for laboratory 10 were both excellent (>90%). Laboratories 5 and 6 had low specificities (<10%) based on originally submitted results, but showed large improvements with both data standardization and with pigeon/DNQ reclassification. Only the specificity at Laboratory 5 remained below 90% after data analysis standardization.

Discussion

The MST methods evaluated in this study covered a range of conventional end-point PCR, SYBR Green qPCR, and Taqman qPCR approaches. All gull MST methods tested demonstrated good average sensitivity ranging between 81 to 100% (Table 2, standardized analysis and original results). Some of the assays showed a greater degree of non-target cross-reactivity than previously reported for gull markers based on *C. marimammalium* 16S rRNA gene sequences, with average specificity ranging from 37 to 91% (Table 2, standardized analysis and original results). The majority of cross-reactivity was near-DNQ range, with a large separation (several orders of magnitude) between gull and non-gull samples. Pigeon feces were an exception, giving amplification at levels as high as or higher than that of gull feces.

DNQ and near-DNQ values were found throughout the various participating laboratories. It might be suspected that such DNQ values may represent cross-contamination or non-specific amplification. However, it is believed that such DNQ values do not necessarily indicate cross contamination. Rather, this study interpreted such values to indicate that there is some target DNA in the PCR reaction at a concentration that is below the lowest standard that was run on the standard curves. A direct interpretation is that samples that are DNQ are detected but not quantifiable. It is only after a close examination of various blanks and controls (QC samples), that a DNQ may be disregarded. In fact, if controls have unexpected amplification, then results within the ROQ might also have to be discarded. This must be determined on a case by case basis. Often, the treatment of DNQ by researchers in this field is not described transparently in manuscripts. It would be helpful if the community could come to a consensus for the treatment of these types of assay results. A full discussion of this topic

is beyond the scope of this paper, but it is discussed in more detail by Stewart *et al.* (2013).

Based on our assessment of the QC samples in this study, we found no justification for disregarding all the DNQ samples, thus retained them and interpreted the results directly. Results for filter blanks and other controls analyzed at the core labs are discussed in the overview paper (Boehm *et al.* 2013). QC of all processing controls (extraction controls, no template PCR controls, etc.) was left up to the individual laboratories to check before submitting their data. While most DNQ values could be interpreted directly based on QC values, it cannot be ruled out that some of the poor specificities observed in some laboratories might be due either to some low level contamination or to some cross-reactivity from more sensitive detection at some labs. In the case of singleton samples, 100% of the source is from a single host animal type and there should not be any low level gull target in these non-gull samples. Therefore, near-DNQ in non-gull singleton samples may likely be due to low-level contamination or to some degree of non-specific amplification. Both scenarios are possible. In general, the filter blank results for single-host qPCR assays indicated only very limited potential contamination, although 8 of 19 filter blank control reactions were positive when assayed with the Gull2Taqman assay. Also, near-DNQ cross-reactivity might have been seen by some labs due to differences in quenching chemistry of the probes used. While most labs had probes synthesized with the quencher TAMRA, some labs used a commercial ZEN quencher (Integrated DNA Technologies, Coralville, IA). This new quencher is supposed to substantially increase sensitivity on the low end of the standard curve, but that increased probe sensitivity may have come at the cost of increased prevalence of DNQ and near-DNQ values that hurt the evaluation of assay specificity.

Based on sequencing of amplicons from SIPP Study samples, sequences nearly identical to *C. marimammalium* were detected from pigeon feces using both Gull2 and LeeSeaGull primers, suggesting that *C. marimammalium* can inhabit the pigeon gut, and that some pigeons can be true positives for *C. marimammalium*–based MST assays. From Figure 1, it appears that ruddy turnstone is also a true positive for this *C. marimammalium* target, although that species was not tested in this study. However, it must again be emphasized that the prevalence, duration, and geographic distribution of this *C. marimammalium* target among non-gull bird populations in not well understood, but that it may actually be quite rare and potentially only associated with non-gull shorebirds that closely co-occur with gulls such that they share gut microbiota (and perhaps only transiently so). The ecology of this target organism must be better understood to address these questions. Although this could be a confounding factor if the desire is for a "gull-only" assay, in many scenarios it might not be necessary to distinguish between pigeon and gull contamination for MST applications. Both of these birds can have a substantial impact on public health and water quality at recreational beaches.

If evaluated solely as a "gull-only" detection assay, then the specificity of these *Catellicoccus*-based MST assays was not acceptable with these particular SIPP samples. However, the performance for all these *C. marimammalium* MST methods improved when detection of pigeon was considered as a true positive for these assays. It should be noted that the pigeon feces were collected only in California; therefore, extending the geographic distribution of this study is recommended (Stewart *et al.* 2013). In addition, extending the types of bird feces tested would be worthwhile. It is possible that *C. marimammalium* from gulls could be acquired by other birds living in proximity to gull colonies through coprophagy or by drinking gull contaminated water. A similar phenomenon was observed with cranes and snow geese co-inhabiting in roosting areas. In this case, species closely related to *Catellicoccus* were present in both types of animals (Ryu *et al.* 2012), whereas this bacterial group was not detected in geese feces or in geese impacted waters that were not also frequented by gulls (Lu *et al.* 2012). Significant mismatches of the primers and the probes were observed with corresponding sequences of *Catellicoccus*-like species obtained from clone fecal libraries of non-gull avian species (Figure 7), suggesting that the both TaqMan-based gull-specific assays tested here are specific to *C. marimammalium*. Moreover, another *Catellicoccus*-based assay (not tested in this study), known as GFC, was recently developed to target only gull feces as well. This GFC assay cross-reacted to a small extent to goose and duck feces (3 of 106 and 4 of 76 samples, respectively) but to none of the pigeon samples tested (n = 13; Green *et al.* 2012). Altogether, *C. marimammalium* has only rarely been detected in some avian species whereas seagull is the most dominant host. Consequently, the *Catellicoccus*-based gull markers

tested in this study are still a useful tool for monitoring seagull fecal contamination, despite potential but likely rare occurrance in some non-gull shorebirds at some locations. Additional studies are needed to determine if gull signals detected in non-gull hosts is due to the possibility of other hosts as transitional habitats of *C. marimammalium*, particularly when non-primary hosts are feeding/roosting during their migratory patterns (stopovers) in areas frequented by gulls. Such a scenario was recently observed with cranes and snow geese with *Catellicoccus* spp. (Ryu *et al.* 2012). In such cases, host-specific assays can still be used as a more general assay, but not to discriminate one avian host over another during overlapping times.

Standardized data analysis improved the performance characteristics of the Taqman assays but not the SYBR Green assay (Table 2). Data standardization across laboratories for the Gull2SYBR assay was more challenging compared to the TaqMan assays. A standardized format for melt curve interpretation was not formulated and therefore the definition and assessment of metrics such as DNQ (when made) were left to the judgment of each laboratory that ran the samples. Likewise, although standardization might improve performance of the Gull2Endpoint PCR assay, it was beyond the scope of this study to recommend methods of standardized gel interpretation.

Normalizing to either the concentration of general Bacteroidales or to DNA mass improved the apparent performance of both the Gull2Taqman and the LeeSeaGull assays versus normalization to enterococci (Figures SI-2 through SI-19). Gulls tend to have high concentrations of enterococci in their feces and low concentrations of Bacteroidales. Normalizing to either of these metrics skewed the non-target data one way or the other in relation to gull and pigeon data. To the authors' knowledge, no one has yet shown a consistent relationship between the relative abundance of enterococci, *C. marimammalium*, and Bacteroidales in the feces of gulls or other birds. Therefore, for this particular study of fecal samples, normalizing to DNA mass was found to be a preferable approach for comparing these gull assays. In this case, normalization of the data was necessary because of the highly variable amounts of feces that were added to each filter. Normalization when considering environmental samples adds additional complexity due to the varying contributions of Bacteroidales and *Enterococcus* from other fecal sources. It should be recognized that all the methods of normalization were problematic in their own way. In the case of using DNA mass, there would certainly be expected differences in the efficiency and quality of DNA that was extracted by different laboratories, especially when using different extraction kits. Also, normalizing to DNA mass for environmental samples may be problematic due to DNA sources from non-target organisms that may be present in substantial abundance.

Assay performance was significantly improved under the following analysis criteria: data analysis was standardized across laboratories to the same definition of ND and LLOQ (and the corresponding DNQ and ROQ assessments), pigeon was considered a true positive, and DNQ values counted as negative. Under these conditions, all the tested gull methods achieved sensitivity and specificity of >80%, meeting the criteria defined by Boehm *et al.* (2013), except for Gull2SYBR which showed a decrease in sensitivity under these conditions (Table 2). Under these analysis conditions, the best average performance was demonstrated by the Gull2Taqman assay (92% sensitivity, 96% specificity) and the LeeSeaGull assay (100% sensitivity, 86% specificity; Table 2).

In general, there was no consistency across laboratories for cross-reactivity with non-target samples (except pigeon). Potential sample contamination could not be ruled out (Boehm *et al.* 2013, Layton *et al.* 2013, SI). The majority of the apparent cross-reactivity was observed at concentrations near or below the DNQ threshold, while the amplification of true positive target was orders of magnitude higher. Similarly to that observed by Layton *et al.* (2013), the interpretation of DNQ as a negative resulted in significant improvement of assay performance. The impact was observed with both binary and quantitative results, and it was particularly important with low target concentration and DNQ samples. In any case, given the general separation between target and nontarget samples (Figures 2 - 7), it is reasonable to assume that all of these assays would be useful under scenarios in which fecal contamination from gulls was relatively high and non-targeted host fecal contamination was relatively low.

Overall, these results support other observations that LOD and LLOQ calculations are important to the interpretation of assay performance (Layton *et al.* 2013, Raith *et al.* 2013, Stewart *et al.* 2013). For example, an 80% criterion was chosen here and the LLOQ value was calculated as the average C_t of the lowest standard included in the standard curve

with outliers removed (Supplemental Information). Consequently, these parameters defined the DNQ range. As discussed in Stewart *et al.* (2013), there are a number of ways to calculate these parameters. Here the LLOQ was set, but LOD values were not defined. In clinical diagnostics, however, the LOD calculation typically employees a 95% criterion and the LLOQ essentially is the LOD value raised by some criterion (e.g., variability in the low standard, e.g., 2 standard deviations; Burd 2010). Such calculations used here could have effectively raised the DNQ criteria, with the consequence of increasing assay specificity (Table 2).

Quantitative source identification of bird fecal contamination is a critical need for water quality managers. This work provided a valuable first step in assessing the performance of these MST methods under inter-laboratory conditions. Future studies should focus on extending the geographical and species range of challenge samples, improving the understanding of the ecology and host prevalence of the *C. marimammlium* target, determining assay performance in different environmental matrices at realistic environmental target concentrations, and further testing performance with real environmental samples.

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Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_523_539SI.pdf.