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

Christopher D. Sinigalliano [a,*], Jared S. Ervin [b,c], Laurie C. Van De Werfhorst [b,c], Brian D. Badgley [d,r], Elisenda Ballesté [e], Jakob Bartkowiak [a,f], Alexandria B. Boehm [g], Muruleedhara Byappanahalli [h], Kelly D. Goodwin [a], Michèle Gourmelon [i], John Griffith [j], Patricia A. Holden [b,c], Jenny Jay [k], Blythe Layton [j], Cheonghoon Lee [l], Jiyoung Lee [l,m], Wim G. Meijer [e], Rachel Noble [n], Meredith Raith [j], Hodon Ryu [o], Michael J. Sadowsky [d], Alexander Schriewer [p], Dan Wang [g], David Wanless [a,f], Richard Whitman [g], Stefan Wuertz [p,q], Jorge W. Santo Domingo [o]

[a] National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratory, 4301 Rickenbacker Causeway, Miami, FL 33149, USA
[b] Bren School of Environmental Science & Management, University of California, Santa Barbara, CA, USA
[c] Earth Research Institute, University of California, Santa Barbara, CA, USA
[d] BioTechnology Institute, University of Minnesota, St. Paul, MN, USA
[e] Conway Institute, UCD School of Biomolecular and Biomedical Science, University College Dublin, Dublin, Ireland
[f] Cooperative Institute for Marine and Atmospheric Studies, University of Miami, Miami, FL, USA
[g] Environmental and Water Studies, Department of Civil and Environmental Engineering, Stanford University, Stanford, CA, USA
[h] U.S. Geological Survey Great Lakes Science Center, Lake Michigan Ecological Research Station, Porter, IN, USA
[i] Laboratoire de Microbiologie, MIC/LNR, Département Ressources Biologiques et Environnement, Unité Environnement, Microbiologie et Phycotoxines, Ifremer, ZI Pointe du diable, Plouzané, France
[j] Southern California Coastal Water Research Project, Costa Mesa, CA, USA
[k] Department of Civil and Environmental Engineering, University of California Los Angeles, Los Angeles, CA, USA
[l] Division of Environmental Health Sciences, College of Public Health, Ohio State University, Columbus, OH, USA
[m] Department of Food Science & Technology, Ohio State University, Columbus, OH, USA
[n] Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, NC, USA
[o] U.S Environmental Protection Agency, Microbial Contaminants Control Branch, National Risk Management Research Laboratory, Cincinnati, OH, USA
[p] Department of Civil and Environmental Engineering, University of California Davis, Davis, CA, USA
[q] Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University, Singapore
[r] Department of Crop and Soil Environmental Sciences, Virginia Tech, Blacksburg, VA, USA

Abbreviations: FIB, fecal indicator bacteria; SIPP, Source Identification Protocol Project; MST, Microbial source tracking; TMDL, total maximum daily load; QMRA, quantitative microbial risk assessment; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; ROQ, range of quantification; LLOQ, lower limit of quantification; DNQ, detected but not quantifiable; ND, not detected; C_t, cycle threshold; CV, coefficient of variation; CFU, colony forming unit; QC, quality control.

* *Corresponding author.* Tel.: +1 305 361 4384; fax: +1 305 361 4447.
E-mail addresses: christopher.sinigalliano@noaa.gov, sinigalliano@yahoo.com (C.D. Sinigalliano).

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ABSTRACT

Here we report 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 gull fecal contamination in coastal environments. The methods included a conventional end-point PCR method, a SYBR® Green qPCR method, and two TaqMan® qPCR methods. Different techniques for data normalization and analysis were 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 measured 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 were found in this study to also detect feces from other birds, particularly feces composited from pigeons. Sequencing efforts showed that some pigeon feces from California contained sequences similar to *C. marimammalium* found in gull feces. These data suggest that the prevalence, geographic scope, and ecology of *C. marimammalium* in host birds other than gulls 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.

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1. 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 *Escherichia coli* are used to monitor microbial water quality. FIB are found also 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 (Graczyk et al., 1998; Quessy and Messier, 1992; Lévesque et al., 2012; 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., 2012). 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 bacteria are widespread in waterfowl. Overall, several bird MST assays target a similar region of the 16S rRNA gene of *C. marimammalium*. The primer and probe hybridization target sites of these various assays in relation to a reference *C. marimammalium* sequence is shown in the Supplemental Material (Fig. S1).

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 North America, South Africa, and Europe (Ryu et al., 2012; Gourmelon, unpublished data).

One limitation of SYBR® Green qPCR assays is the difficulty of quantifying the targeted genetic sequence if spurious amplification occurs. To increase specificity, the Gull2 assay was 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 Gull2-Taqman assay has been 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 was positively correlated to independent camera-system measurement of gull abundance at beaches (Solo-Gabriele et al., 2011).

In addition to the Gull2 assays described above, this study also includes a new qPCR assay for detection of gull feces, the LeeSeaGull assay (Lee et al., 2013). The LeeSeaGull assay is based on detection of the same target region of *C. marimammalium* as the Gull2 assay, but uses a different primers/probe set which amplifies a smaller PCR product internal to that which is amplified by the Gull2 assays (Fig. 7 and Fig. S1).

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 (Ebentier et al., 2013).

The work reported here was conducted by 11 different participating laboratories. We examined the performance of several gull assays by challenging them to a set of purified DNA samples extracted from single-source and mixed fecal slurries from different animals, sewage, and septage (Boehm et al., 2013). We highlight the findings of this effort and discuss 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 host-specific assay performance are reported elsewhere in this issue (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).

2. Materials and methods

2.1. *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, pigeon fecal samples were collected from 4 regional locations along California. Samples were taken from coastal, recreational, and residential areas at 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 (>20/region) in order to create two pigeon fecal composites. From the combination of all the

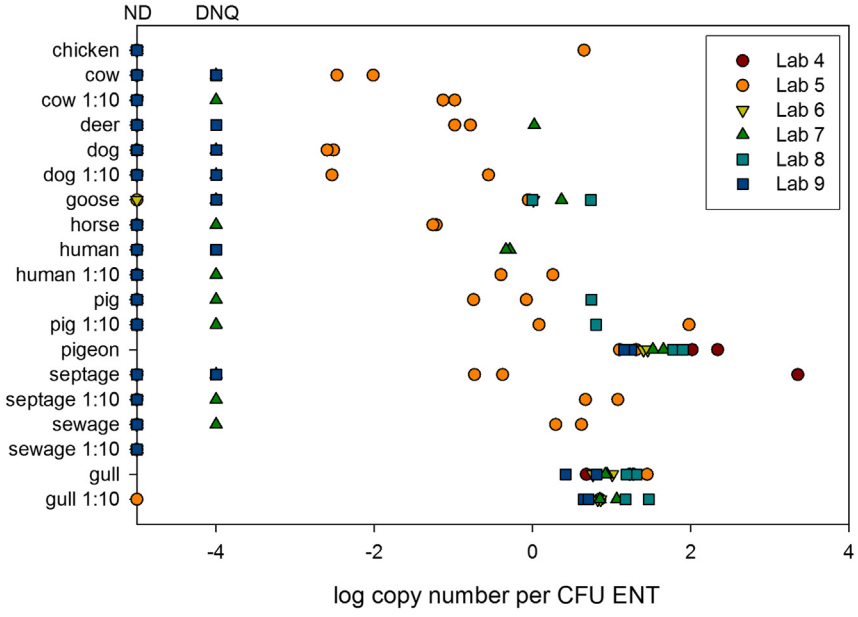

Fig. 1 – Original submitted non-standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by viable enterococci CFU measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

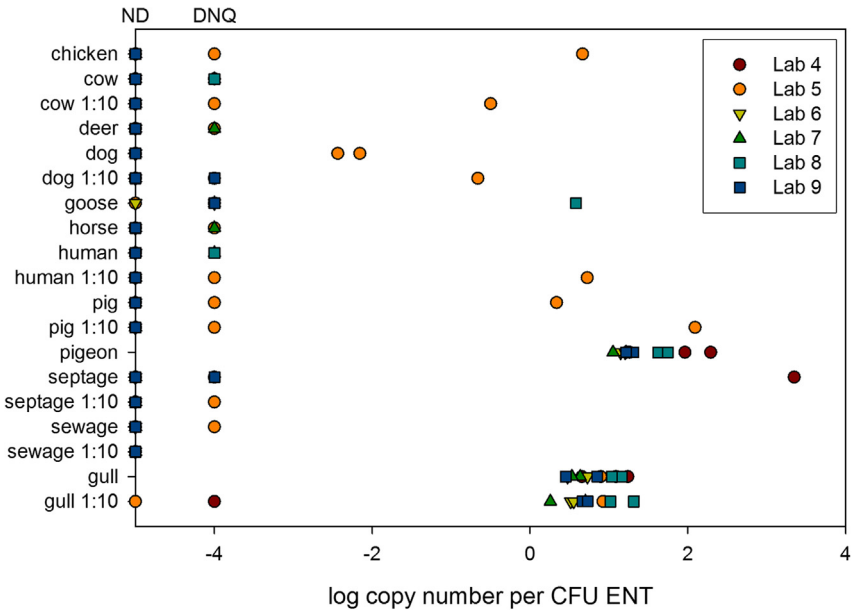

Fig. 2 – Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by viable enterococci CFU measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

various host-source fecal types, thirty-two total types of challenge samples (Table S1) were created from fecal slurries and/or sewage or septage in either "singleton" (individual fecal source) or "doubleton" mixtures (2 fecal sources in 90%:10% ratios by volume). Fifty milliliters 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 1/10th 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 on-line supplemental material 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 (Table S1). In addition, three laboratories tested the LeeSeaGull assay (Table S1), however data from only one of these laboratories was

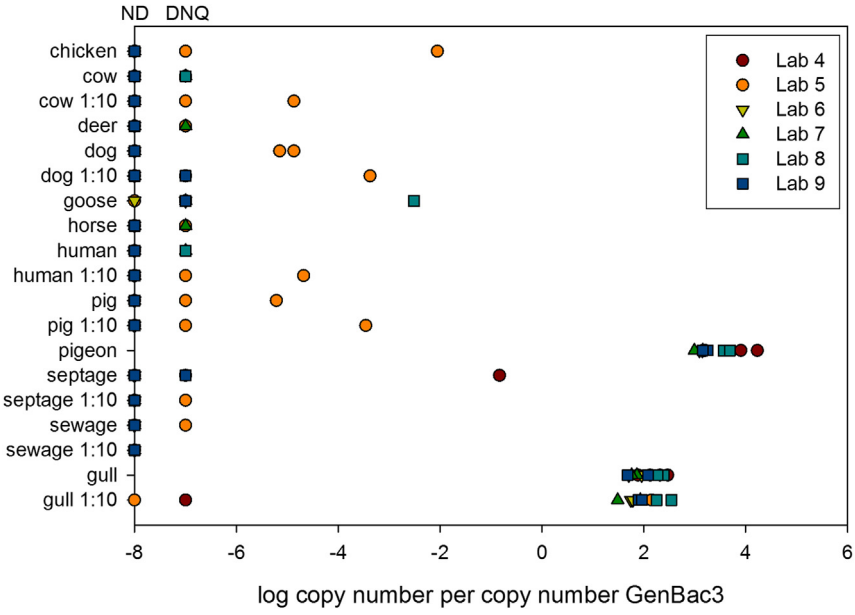

Fig. 3 – Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by *Bacteroidales* genbac3 copy number measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

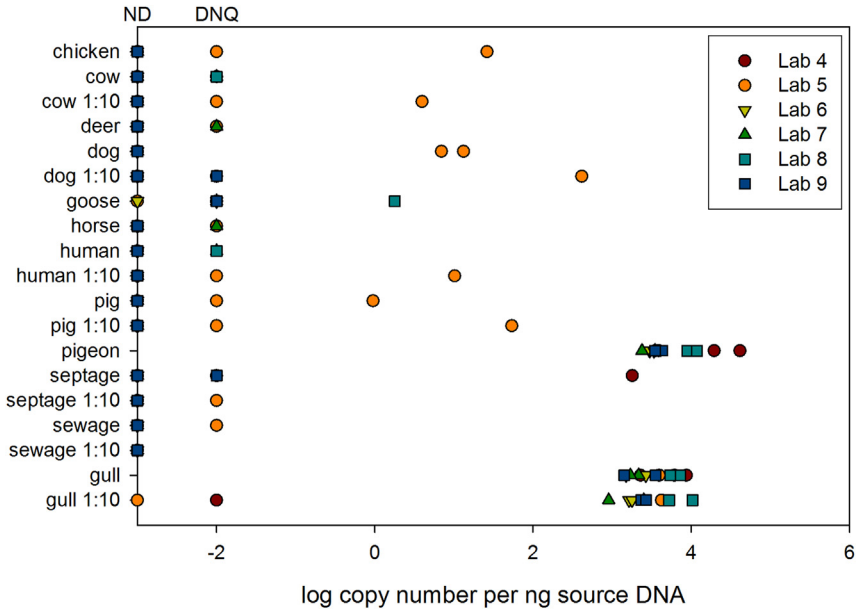

Fig. 4 – Standardized Gull2Taqman assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

presented in the overview paper for this assay (Boehm et al., 2013). Data reported here for the LeeSeaGull assay 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.

2.2. *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 Supplemental Material, Table S3 for details). The additional evaluation of the LeeSeaGull assay utilized DNA from each of the 64 samples. To obtain sufficient volumes for

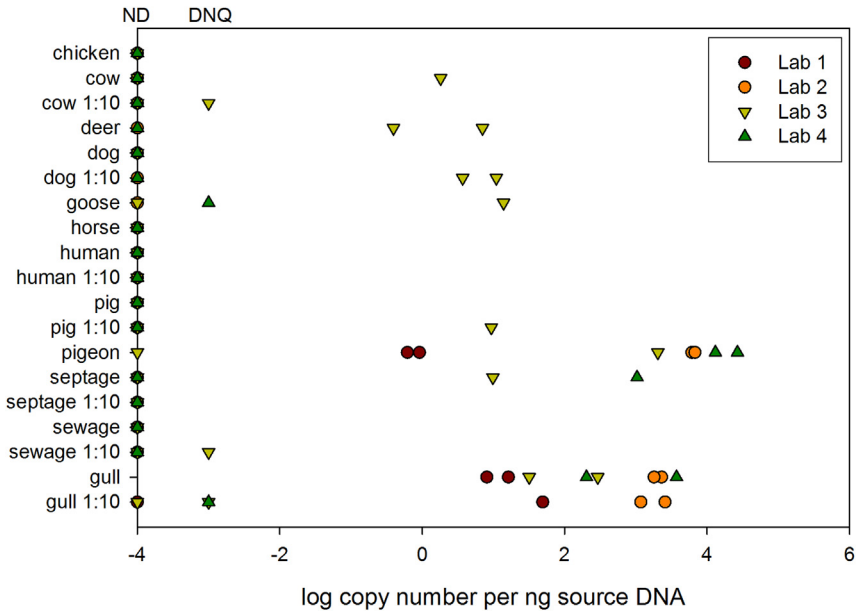

Fig. 5 – Standardized Gull2SYBR assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

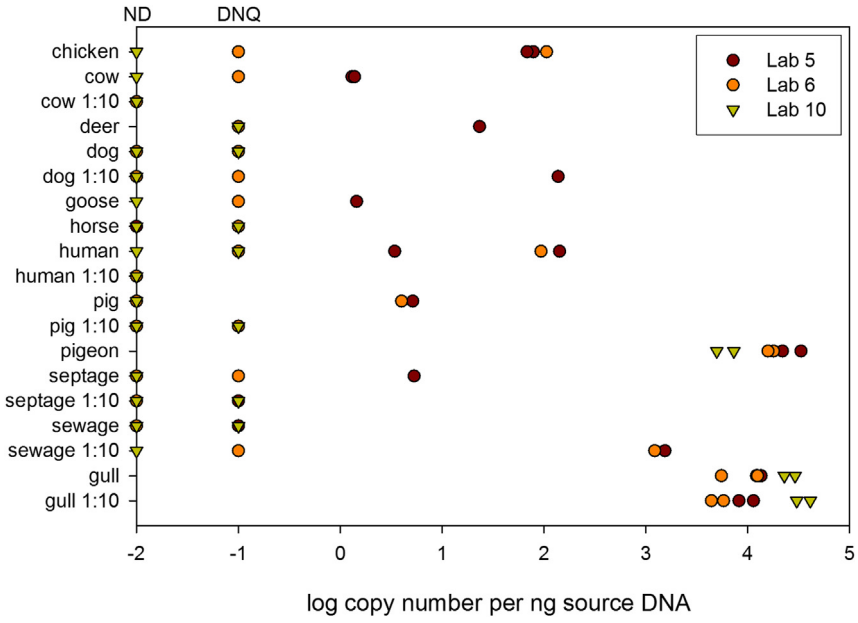

Fig. 6 – Standardized LeeSeaGull assay results from the 38 single-source challenge samples, normalized by ng DNA measured in the sources. ND = not detected. DNQ = detected but not quantifiable.

```
TGCATCGACCTAAAGTTTTGAG                                                                  Gull2 primers/probe
                       AGGTGCTAATACCGCATAATACAGAG      TTCTCTGTTGAAAGGCGCTT             LeeSeaGull primers/probe
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATACAGAG.....TTCTTTGTTGAAAGGCGCTT..... NR_042357_C. marimammalium
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATACAGAG.....TTCTTTGTTGAAAGGCGCTT..... JN394056_Gull
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATACAGAG.....TTCTTTGTTGAAAGGCGCTT..... JN394070_Gull
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATAYAGAG.....TTCTCTGTTGAAAGGCGCTT..... EU181065_Gull
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATACAGAG.....TTCTTTGTTGAAAGGCGCTT..... SIPP_pigeon
TGCATCGACCTAAAGTTTTGAG.....AGGTGCTAATACCGCATAATACAGAG.....TTCTTTGTTGAAAGGCGCTT..... RT0048_Ruddy turnstone
TGCACCGACCTAAGTTTCGGAG.....AGGTGCTAATACCGCATAAATCCTTTT....AAAAGGCTGAAAGGCGCTT-..... JQ015177_Snow goose
TGCATCGACCTAAGTTCTCAGAG....AGGTGCTAATACCGCATAATTCCTTTT....AAAAGGCTGAAAGACGCTT-..... JQ015168_Crane
TGCACCGACCTAAGTTTTTAGAG....AGGTGCTAATACCGCATAATTCCTTTT....AAAAGGCTGAAAGGCGCTT-..... JQ015169_Crane
TGCACCGACCTAAGTTTCGGAG.....AGGTGCTAATACCGCATAAATCCTTTT....AAAAGGCTGAAAGGCGCTT-..... JQ015171_Crane
TGCATCAACCAAACAATCGGAG.....AGGTGCTAATACCGCATAAGAGCGAT.....TTATTGTTTAAAAGGCGCTT..... RK0144_Red Knot
TGCATCGACCTAAGTTAGAAGAG....AGGTGCTAATACCGCATAACACTAAA.....TTTTTAGTTGAWAGGCGCTC..... RK0566_Red Knot
TGCATCGACCTAAGTTAGAAGAG....AGGTGCTAATACCGCATAACACTAAA.....TTTTTGGTTGAAAGGCGCTT..... SPS0217_Semipalmated sandpiper
TGCACTAACTTAAGTTAGAAGAG....AGGTGCTAATACCGCATAATACTTGA.....TTTCAAGTTGAAAGGCGCTT..... SPS0267_Semipalmated sandpiper
|--------|---------|--.....-----|---------|---------|.....|--------|---------|
41       50        60          130       140       150   161      170       180

                                CTGAGAGGGTGATCGGCCACATTGGGACT     TAGTAACTGCTCGCTCTTTGAC    Gull2 primers/probe
     TAGACGGTGAGGTAACGGC                                                                  LeeSeaGull primers/probe
.....TAGACGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGCTCGCTCTTTGAC NR_042357_C. marimammalium
.....TAGTTGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACACTGGGACT.....TAGTAACTGCTCGCTCTTTGAC JN394056_Gull
.....TAGACGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGCTCGCTCTTTGAC JN394070_Gull
.....TAGACGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGCTCGCTCTTTGAC EU181065_Gull
.....TAGACGGTGAGGTAACGGC,,,,,CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGCTCGCTCTTTGAC SIPP_pigeon
.....TAGACGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGCTCGCTCTTTGAC RT0048_Ruddy turnstone
.....TAGTCGGTGGGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTCCTTGAC JQ015177_Snow goose
.....TAGTCGGTGGGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTCCTTGAC JQ015168_Crane
.....TAGTCGGTGGGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTTTTTGAC JQ015169_Crane
.....TAGTCGGTGGGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTCCTTGAC JQ015171_Crane
.....TAGTCGGTGGGGTAAAGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGATTCTACCTTGAC RK0144_Red Knot
.....TAGTCGGTGGGGTAAAGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGATTCTACCTTGAC RK0566_Red Knot
.....TAGTCGGTGGGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTCCTTGAC SPS0217_Semipalmated sandpiper
.....TAGACGGTGAGGTAACGGC.....CTGAGAGGGTGATCGGCCACATTGGGACT.....TAGTAACTGGCTTCTCCTTGAC SPS0267_Semipalmated sandpiper
.....---|---------|-----.....-------|---------|---------|--....|---------|---------|--
        220       230             270       280       290   430       440       450
```

Fig. 7 – Comparison of *Catellicoccus marimammalium* and *Catellicoccus*-like clone sequences from different waterfowl and shorebirds. 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).

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.

2.3. PCR and MST target analysis of Catellicoccus marimammalium

Primer and probe oligonucleotide sequences for the tested assays are shown in Table S4, and their hybridization target locations are shown in a sequence alignment relative to a reference *C. marimammalium* 16S rDNA gene sequence in Fig. S1. Protocols used in different laboratories for each assay were similar but variations existed between some laboratories with regard to reagents, cycling platforms, probe quencher chemistries, and in some cases cycling conditions. Briefly, protocols for the Gull2SYBR qPCR and Gull2Endpoint PCR assays were based on Lu et al. (2008), 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 Supplemental Material, with protocol variations by laboratory detailed in Table S5.

2.4. Sequencing verification of PCR amplicon identity from pigeon fecal samples

Due to a consistent high level of amplification observed with the pigeon fecal samples tested in this study, amplicons generated from both the Gull2Taqman and the LeeSeaGull assays for the two duplicate pigeon fecal challenge samples were sequenced with the Gull2 and LeeSeaGull assay primers, respectively. Amplicons were purified with commercial PCR purification kits, and sequenced using the ABI BigDye Terminator (v3.1) cycle sequencing chemistry with an ABI Prism 3730 DNA Analyzer, generating sequences from both forward and reverse primers. The sequence data from these composite pigeon fecal samples were compared with other *Catellicoccus* sequences provided by the laboratory of Dr. Jorge Santo Domingo as part of a separate, on-going study (Grond et al., unpublished data). A multiple-sequence alignment was generated which included the primers and probes from the assays, the *C. marimammalium* reference sequence, and *Catellicoccus* and *Catellicoccous*-like consensus sequences from a variety of shorebirds, including gulls, pigeons, cranes, snow geese, ruddy turnstones, red knot sandpiper, and semipalmated sandpiper. More details on the sequencing can be found in the Supplemental Material.

2.5. 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 Supplemental Material 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 (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 regarding designations of within the range of quantification (ROQ), detected but not quantifiable (DNQ), or not detected (ND) (see Supplemental Material).

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.

3. 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 (Figs. 1–6). 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 (Figs. 1–6). Due to overlapping symbols, the extent of the DNQ results may be difficult to observe in these figures; therefore Tables 3–5 are presented to clarify the total number and percentage of challenge samples that were not detected, DNQ, or within the range of quantitation for the Gull2Taqman, Gull2SYBR, and LeeSeaGull qPCR assays.

3.1. Gull2Taqman assay

The non-standardized data as submitted by each of the six laboratories performing the Gull2Taqman assay are shown in Fig. 1, whereas Fig. 2 shows the same plot using standardized data analysis (see Section 2.4). Standardization of data analysis (Section 2.4 and Supplemental Material) removed much of the cross-reactivity observed (Figs. 1 and 2). Most non-target

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	−3.70	41.7	0.98	86.5	36.2	31
	6	−3.43	41.2	1.00	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	−3.40	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	1.00	95.5	38.8	97
	6	−3.42	44.1	1.00	95.9	36.4	180
	10	−4.26	50.0	0.99	71.7	36.6	1402

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 Supplemental Material). 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). 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 (Fig. 3). 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 (Figs. 1 and 2). 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 2 – %Sensitivity (sens) and %specificity (spec) for each assay with varying sets of defined analysis parameters.[a]

Assay	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	92.3	100	67.3	100	67.3	100	69.2	91.7	67.0
Standardized analysis	66.7	92.2	91.7	9.6	100	92.3	100	80.8	100	80.8	100	82.7	93.1	73.1
Standardized analysis, pigeon+, DNQ–	57.1	98.0	92.9	78.0	100	100	100	100	100	98.0	100	100	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	96.2	91.7	80.8	83.3	90.4					89.6	90.9
Standardized analysis	50.0	96.2	100	96.2	91.7	76.9	83.3	90.4					81.3	89.9
Standardized analysis, pigeon+, DNQ–	50.0	100	100	100	78.6	84.0	64.3	98.0					73.2	95.5
	Lab 5		Lab 6		Lab 10								Average	
LeeSeaGull	Sens	Spec	Sens	Spec	Sens	Spec							Sens	Spec
Original submitted results	100	7.7	100	9.6	100	94.2							100	37.2
Standardized analysis	100	40.4	100	32.7	100	75.0							100	49.4
Standardized analysis, pigeon+, DNQ–	100	68.0	100	90.0	100	100							100	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	58.8	100	94.2	83.3	96.2					85.4	85.4
Pigeon+	64.3	96.0	100	61.2	100	98.0	85.7	100					87.5	88.8

[a] 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 Material.

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	Gull2Taqman		
	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 (Fig. 4). 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.

3.2. *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) (Fig. 5). 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.

3.3. *LeeSeaGull assay*

Data analysis was standardized for the LeeSeaGull assay and data were normalized to DNA mass in the single-source samples (Fig. 6). Two of the three laboratories used aliquots of the same pooled DNA (see section 2.2). 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)	1 (17)
Goose	2 (33)	3 (50)	1 (17)
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.

3.4. *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.

3.5. *Sequencing of amplicons from Gull2Taqman and LeeSeaGull assays*

Analysis of clone libraries developed using the Gull2 primers showed that the sequences derived from the composite pigeon fecal samples of this study were identical to that of a reference *C. marimammalium* sequence (NCBI accession number: NR_042357). For the LeeSeaGull primers, there was a single base pair mis-match to this reference sequence out of an 112 bp amplicon. Thus pigeon fecal amplicons from both of these gull assays showed >99% sequence identity to a reference *C. marimammalium* sequence. Fig. 7 shows that sequences observed from gull, pigeon, and ruddy turnstone feces were nearly identical to the *C. marimammalium* reference sequence (>99% identity), whereas other shorebirds, crane, and snow goose feces contained 16S rRNA gene sequences closely related to *Catellicoccus* spp., but which differed significantly from *C. marimammalium* (≤95% identity). Consistent with these findings, in the on-going investigation that generated these additional sequences, *C. marimammalium* qPCR signals were detected in seagull, pigeon, and ruddy turnstone feces, but not in feces from the other bird species tested (Grond et al., unpublished data). We were not able to determine the general distribution of *C. marimammaium* in pigeons because the samples in the SIPP study reported here were composited from multiple individuals from a variety of geographic areas.

3.6. *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 ("standardized analysis", Table 2). 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 ("standardized analysis, pigeon+, DNQ−", Table 2). Additional sensitivity and specificity results are shown in Table S6, 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 ("Pigeon+," Table 2). 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 S7.

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.

4. 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% and 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.

Additional work was performed for this study so that all assays were evaluated by more than one laboratory. When evaluated solely as a "gull-only" detection assay, the specificity of all the *Catellicoccus*-based MST assays fell below the criteria established for the SIPP study. However, the performance for all the assays improved when detection of pigeon was considered as a true positive for these assays. Sequences identical or nearly identical to *C. marimammalium* were detected from Gull2 and LeeSeaGull assay amplicons from pigeon feces, demonstrating that the proper target was present in the amplicons. Some filter blanks (Boehm et al., 2013) produced positive signal, so contamination during filter preparation cannot be ruled out. However, these data observed in the context of the additional sequence data (Fig. 7) suggests that at least some pigeons can be true positives for *C. marimammalium*. However, the prevalence, duration, and geographic distribution of *C. marimammalium* among non-gull bird populations are not well understood and could not be fully addressed here because the fecal samples were composites.

In contrast to the results reported here, another *Catellicoccus*-based qPCR test, known as the GFC assay (not evaluated in this study), was recently developed to target gull feces. This GFC assay was reported to cross-react with goose and duck feces to a small extent (3/106 and 4/76 samples, respectively) but with none of the pigeon samples tested ($n = 13$) (Green et al., 2012). Taken all together, results suggest that seagull is the dominant host for *C. marimammalium*, but sometimes may be detected in the feces of non-gull species. It should be noted that the pigeon feces in this SIPP study were collected only in California and were analyzed in composite samples; extending the geographic distribution of this study is recommended (Stewart et al., 2013).

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). Additional studies are needed to determine if non-gull hosts can harbor *C. marimammalium* due to contact with gulls, including transitional exposure during migration. Such a scenario could be confounding if the desire is a "gull-only" assay; however, many MST applications may not require a distinction between pigeon and gull contamination because both birds can impact water quality at recreational beaches.

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 (Figs. S2–S19). 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 our 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, we contend that for this particular study of fecal samples, normalizing to DNA mass was 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 enterococci from other fecal sources. It should be recognized that all the methods of normalization were problematical 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).

It is possible that some of the DNQ results could be attributed to cross contamination, perhaps during filter preparation. Some filter blanks (8/19) showed positive signal for the Gull2Taqman assay (Boehm et al., 2013) and may have occurred for the human marker (Layton et al., 2013,

Supplemental Material). In general, there was no consistency across laboratories for cross-reactivity with non-target samples (except pigeon). 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 (Figs. 1–6), 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, Ervin 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 Material). 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 employs a 95% criterion and the LLOQ essentially is the LOD value raised by some criterion (e.g., variability in the low standard; for example, 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.

5. Conclusions

- This study evaluated four MST assays to detect gull fecal contamination. This paper presents additional laboratory findings and new data analysis that were not represented in the overview paper.
- Standardized data analysis (standardization of LLOQ, ND, and DNQ definitions across laboratories) significantly improved performance, with all assays meeting a threshold of >80% sensitivity for average across-laboratory sensitivity. Under additional data analysis considerations, the Gull2Endpoint, Gull2Taqman, and LeeSeaGull assays demonstrated average across laboratory specificity of >80% (but only if pigeon was considered a true positive). These findings highlight the need to provide defined guidelines for data analysis as part of protocol standardization.
- All assays detected pigeon feces with sensitivity and specificity similar to that observed for gull feces and DNA sequencing confirmed *Catellicoccus* target sequences from pigeon feces, indicating that pigeon samples in this study could also be true positives for the targeted fecal indicator. Additional testing is needed before extending these results to other geographic areas.
- Other than pigeon, most cross-reactive samples returned values near or below the LLOQ. Although potential contamination could not be ruled out, reclassification of DNQ results as true negatives increased assay performance, suggesting that DNQ calculations may have been below the true limit of detection of the assay and indicating that further efforts to validate LOD values are warranted.
- Additional studies are recommended to further test these assays with samples from more bird species, extended geographical range, and different environmental matrices.
- Across-laboratory assay performance was improved when data were normalized by DNA mass measured in the sources rather than by abundance of viable enterococci, indicating that further evaluation is needed on how to bring these MST tools to bear on the problem of source attribution.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.02.059.

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