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Performance evaluation of canine-associated *Bacteroidales* assays in a multi-laboratory comparison study

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ABSTRACT

The contribution of fecal pollution from dogs in urbanized areas can be significant and is an often underestimated problem. Microbial source tracking methods (MST) utilizing quantitative PCR of dog-associated gene sequences encoding 16S rRNA of *Bacteroidales* are a

Abbreviations: cp, gene copy numbers; Ct, cycle threshold; DNQ, detected but not quantifiable; FIB, fecal indicator bacteria; LLOQ, lower limit of quantification; LOD, limit of detection; MST, microbial source tracking; ND, not detected; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; ROQ, range of quantification; SIPP, Source Identification Protocol Project; TMDL, total maximum daily load.

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useful tool to estimate these contributions. However, data about the performance of available assays are scarce. The results of a multi-laboratory study testing two assays for the determination of dog-associated *Bacteroidales* (DogBact and BacCan-UCD) on 64 single and mixed fecal source samples created from pooled fecal samples collected in California are presented here. Standardization of qPCR data treatment lowered inter-laboratory variability of sensitivity and specificity results. Both assays exhibited 100% sensitivity. Normalization methods are presented that eliminated random and confirmed non-target responses. The combination of standardized qPCR data treatment, use of normalization *via* a non-target specific *Bacteroidales* assay (GenBac3), and application of threshold criteria improved the calculated specificity significantly for both assays. Such measures would reasonably improve MST data interpretation not only for canine-associated assays, but for all qPCR assays used in identifying and monitoring fecal pollution in the environment.

1. Introduction

Fecal material contributes to microbial pollution at many coastal and freshwater sites. Dog feces, in particular, can account for poor water quality near rivers and beaches, because it contains a significant amount of fecal indicator bacteria (FIB). Loading estimates for canine fecal matter range from 1.5×10^8 to 5.6×10^9 CFU of enterococci/g dry weight, and the loading from one dog fecal event is comparable to 6940 bird fecal events (Wright et al., 2009; Zhu et al., 2011). Dog exercise areas have been shown to impact water quality (Garfield and Walker, 2008), and dog feces is a potential source of pathogens (Bagcigil et al., 2007; Gookin et al., 2007; Houf et al., 2008; Papini et al., 2009; Damborg et al., 2009). In addition, dogs can carry antibiotic resistant bacteria (Damborg et al., 2009; Baptiste et al., 2005; Moodley et al., 2006; Nuttall et al., 2008), and infections between humans and co-habiting dogs appear to be emerging (Weese et al., 2006).

Accurate source identification can be used to address beach and shellfish contamination problems and for Total Maximum Daily Load (TMDL) determinations of allowable FIB levels, as a means to estimate supportable inputs of pathogens into a specific watershed (Hagedorn et al., 2011; Kern et al., 2002). Once sources of contamination have been identified, scientifically and economically sound remediation efforts can be devised. To best aid watershed management, microbial source tracking (MST) markers should provide a quantitative means for fecal source identification as demonstrated previously (Reischer et al., 2006; Lee et al., 2010; Schriewer et al., 2010; Tambalo et al., 2012a). To address this need, MST assays utilizing quantitative PCR (qPCR) to identify fecal contamination from dogs have been developed and utilized in field studies (Kildare et al., 2007; Sinigalliano et al., 2010; Schriewer et al., 2010). The assays discussed here, DogBact (Sinigalliano et al., 2010) and BacCan-UCD (Kildare et al., 2007), both target *Bacteroidales* (Dick et al., 2005a, 2005b), but have not been compared previously. In addition, it is uncommon for multiple laboratories to devote resources towards a round-robin test and there are no examples of qPCR-based methods having been validated in an interlaboratory exercise. For qPCR-based MST methods, only within-laboratory (intra-laboratory) studies have been performed (Shanks et al., 2010a, 2010b).

The objectives of this study were (i) to compare two promising quantitative PCR (qPCR)-based canine-associated assays, BacCan-UCD (Kildare et al., 2007) and DogBact (Sinigalliano et al., 2010), that target genes encoding *Bacteroidales* 16S rRNA in a multi-laboratory comparison study using a single-blinded approach with purified DNA from fecal slurries originating from either one animal or human source (singleton) or two combined sources (doubleton) (Boehm et al., 2013) and (ii) to develop a general procedure for the normalization of source tracking data obtained by qPCR. Eight different laboratories participated in the study, all of which received filters of the same challenge samples. This effort was part of a broader multi-laboratory assessment of MST method performance, the State of California-funded Source Identification Protocol Project (SIPP). Other host targets and aspects of assay performance are reported elsewhere in this issue (Boehm et al., 2013; Layton et al., 2013; Ebentier et al., 2013; Raith et al., 2013; Sinigalliano et al., 2013).

2. Materials and methods

2.1. Preparation of challenge samples and processing

Briefly, sixty-four challenge samples were created by mixing fresh feces, sewage, or septage, in artificial freshwater. Feces used were from chicken, cow, dog, deer, goose, gull, horse, human, pig, and pigeon. This set of sixty-four blind samples was comprised of 19 single-source ("singleton") and 13 mixed-source ("doubleton") samples in duplicate. Each doubleton sample contained human feces, septage, or sewage in combination with one non-human source. Except for sewage (untreated influent from nine treatment facilities) and septage (six septage collection trucks or community systems) a minimum of 12 individual samples were collected across California for each animal type. The challenge samples were filtered (50 mL or 5 mL, see supplementary data) over polycarbonate membrane filters with a diameter of 47 mm diameter and a pore size of 0.4 μm (Isopore, Millipore) and distributed frozen to the participating laboratories. The methods for creation and laboratory analysis of the challenge samples are described in detail elsewhere (Boehm et al., 2013) and in the supplementary data for this study.

Table 1 – Standard curve statistics for canine associated assays with standardized post processing.

Assay	Lab	Slope	y-intercept	R^2	Efficiency[a] (%)	LLOQ (C_t)	LLOQ (cp/rxn)
DogBact	1	−3.46	38.4	0.992	94.7	34.8	11
	2	−3.51	44.8	0.951	92.8	38.2	77
	3	−3.35	41.5	0.990	98.8	37.5	16
	4	−3.23	39.0	0.982	104.0	35.6	11
	5	−3.54	41.5	0.986	91.5	37.6	13
	6	−3.41	46.5	0.997	96.5	38.6	217
BacCan-UCD	1	−3.43	44.7	1.000	95.8	37.9	100
	3	−3.27	45.7	0.997	102.3	38.9	122
	7	−3.60	46.0	0.992	89.5	38.8	100
	8	−3.36	43.0	0.971	98.5	32.7	1205

[a] Efficiency $= -1+10^{(-1/\text{slope})}$.

2.2. *Canine-associated qPCR*

The samples were analyzed for two different dog-associated *Bacteroidales* qPCR assays by eight laboratories from the United States and European Union. Some of the laboratories tested both assays, with the result that the DogBact assay (Sinigalliano et al., 2010) was run by six and the BacCan-UCD assay (Kildare et al., 2007) by four laboratories. Two different nucleic acid extraction methods were used: GeneRite DNA EZ Extraction kit (7) and DNeasy Blood & Tissue Kit (Qiagen) (1) (for further details see supplementary data).

2.3. *Standardized calculation of qPCR standard curves and results*

After obtaining Cycle Threshold (C_t) values, laboratories differed in how final concentrations were calculated and how non-detects (ND), or detected but not quantifiable (DNQ), were defined. Some labs decided not to report DNQ data. To eliminate differences in results based on individual lab treatment, the raw C_t values for challenge samples and standard curves were collected from all laboratories and processed in the same way.

Table 2 – Sensitivity and specificity of assays based on originally submitted and standardized data from either singletons alone or from singletons and doubletons together.

	Laboratory number											
	DogBact						AVG[a]	BacCan-UCD				AVG[a]
	1	2	3	4	5	6		1	3	7	8	
a) Original submitted data												
Singletons, DNQ = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	1.00	**1.00**
Specificity	0.03	0.38	0.	0.85	0.65	0.35	**0.58**	0.68	0.59	0.82	0.71	**0.70**
Singletons, DNQ = positive												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	1.00	**1.00**
Specificity	0.03	0.38	0.68	0.85	0.65	0.35	**0.58**	0.32	0.47	0.82	0.71	**0.54**
With doubletons, DNQ = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	1.00	**1.00**
Specificity	0.02	0.27	0.68	0.82	0.63	0.34	**0.55**	0.66	0.61	0.84	0.63	**0.70**
b) Standardized data												
Singletons, DNQ = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	0.00	**1.00**
Specificity	0.00	0.59	0.91	0.94	0.91	0.79	**0.83**	0.68	0.59	0.79	1.00	**0.69**
With doubletons, DNQ = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	0.00	**1.00**
Specificity	0.00	0.50	0.91	0.95	0.91	0.77	**0.81**	0.68	0.61	0.82	1.00	**0.70**
b1) Log(1/RSD)method												
Singletons, values larger RSD (150%) = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	0.00	**1.00**
Specificity	0.00	0.76	0.94	0.94	0.94	0.88	**0.89**	0.82	0.76	0.88	1.00	**0.82**
b2) Normalized via GenBac3												
Singletons, values smaller Log (0.01) = ND												
Sensitivity	1.00	1.00	1.00	1.00	1.00	1.00	**1.00**	1.00	1.00	1.00	0.00	**1.00**
Specificity	0.71	0.88	1.00	1.00	1.00	0.91	**0.96**	0.91	0.91	0.88	1.00	**0.90**

[a] AVG: average, results from lab 1 (DogBact) and lab 8 (BacCan-UCD) are excluded.

Regression analysis was performed on each individual standard curve to remove outliers; C_t values with a residual value larger than +3 or smaller than −3 were ignored when determining the final standard curve for each lab. The initial calculation of LLOQs using the precision of replicate standard curves resulted in values below those that could be confidently detected as lowest standard; for this reason we chose as a more conservative approach to only consider concentrations that yielded amplification in at least 50% of the replicates. Based on these LLOQs, copy numbers (cp) per reaction were calculated for each lab from the average C_t value of the non-outlier standard replicates at the lowest concentration included in the standard curve (Table 1). Each sample replicate was considered within the range of quantification (ROQ) if the C_t < LLOQ, detected but not quantifiable (DNQ) if the C_t > LLOQ, and not detected (ND) if there was no amplification detected by cycle 40.

Since laboratories also differed in numbers of dilutions and replicates of samples tested, a uniform strategy was applied to standardize the interpretation of results into categories of range of quantification (ROQ), DNQ or ND (for details see supplementary data). Basically, the majority category among replicates defined whether the sample was declared ROQ, DNQ, or ND. For 50:50 situations in replicates between ROQ and DNQ as well as DNQ and ND, both cases were defined as DNQ. Specifically, for samples run in triplicate, C_t values were averaged if 2/3 or 3/3 replicates were within the ROQ. If 2/3 or 3/3 replicates were DNQ, then the sample was considered DNQ. If 2/3 or 3/3 replicates were ND

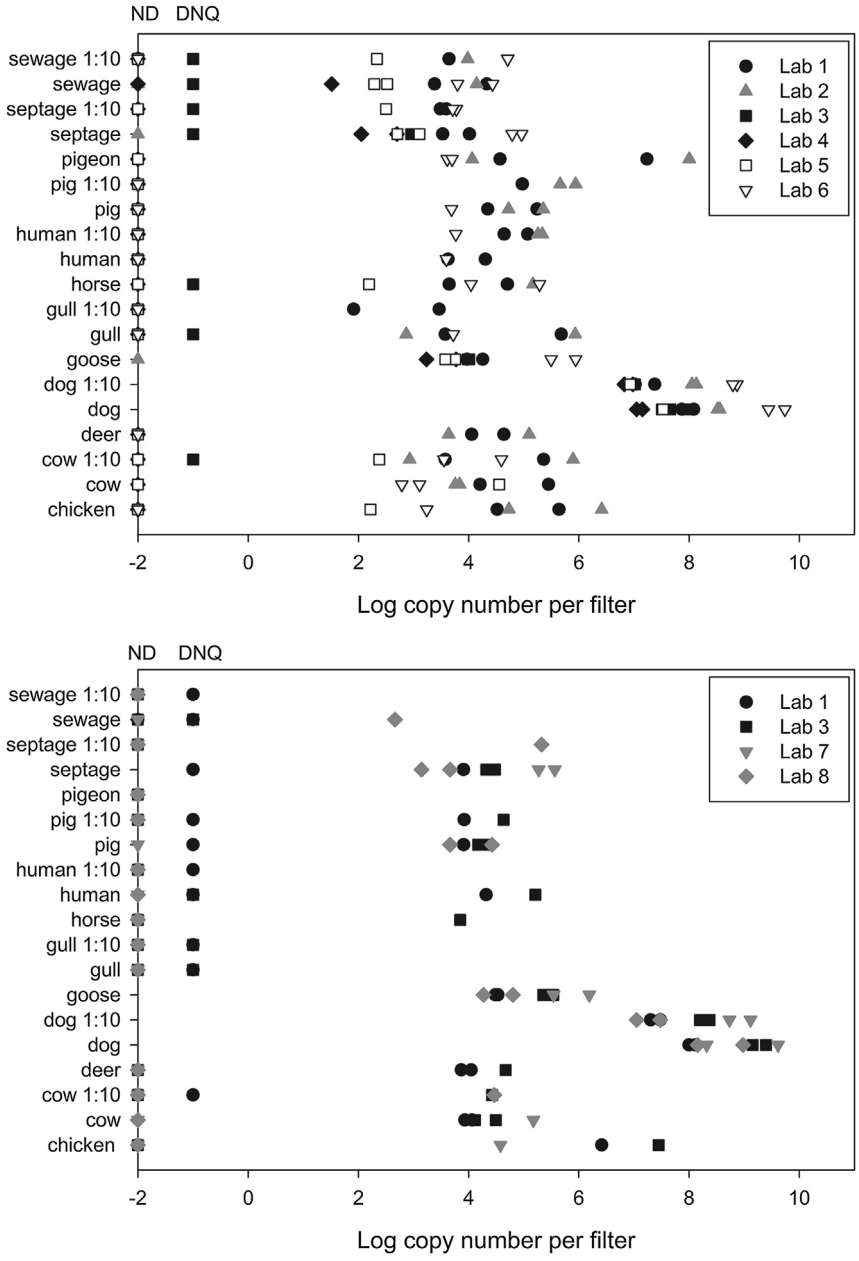

Fig. 1 – Originally submitted results from 38 single-source (duplicate) samples for DogBact (top) and BacCan-UCD (bottom). ND = not detected. DNQ = detected but not quantifiable.

then the sample was considered ND. In the rare case that the replicates contained one each of ROQ, DNQ and ND, then the samples were considered DNQ. For samples run in duplicate, C_t values were averaged if 2/2 replicates were within the ROQ and a single C_t values was used if 1/2 replicates were within the ROQ. If 2/2 replicates were DNQ or 1 DNQ and 1 ND resulted, then the sample was considered DNQ. If 2/2 replicates were ND then the sample was considered ND. For samples run in dilution series all C_t values were considered and, accounting for dilution factors, converted into copy numbers per reaction of undiluted sample template, using each lab's standardized standard curve. These concentrations were averaged and, according to individual LLOQ values, grouped into ROQ, DNQ, and ND. Thus, sample concentrations below LLOQ were considered DNQ. When C_t values were reported at >40 cycles, then they were considered ND.

2.4. *Sensitivity and specificity*

Sensitivity (%) was calculated as the number of challenge samples correctly identified as positive for the host feces divided by the total number of samples that contain the host feces:

$$\text{Sensitivity} = \frac{TP}{(TP + FN)} \quad (1)$$

where TP and FN are true positives and false negatives, respectively.

Specificity (%) was calculated as the number of challenge samples correctly identified as negative for the host feces

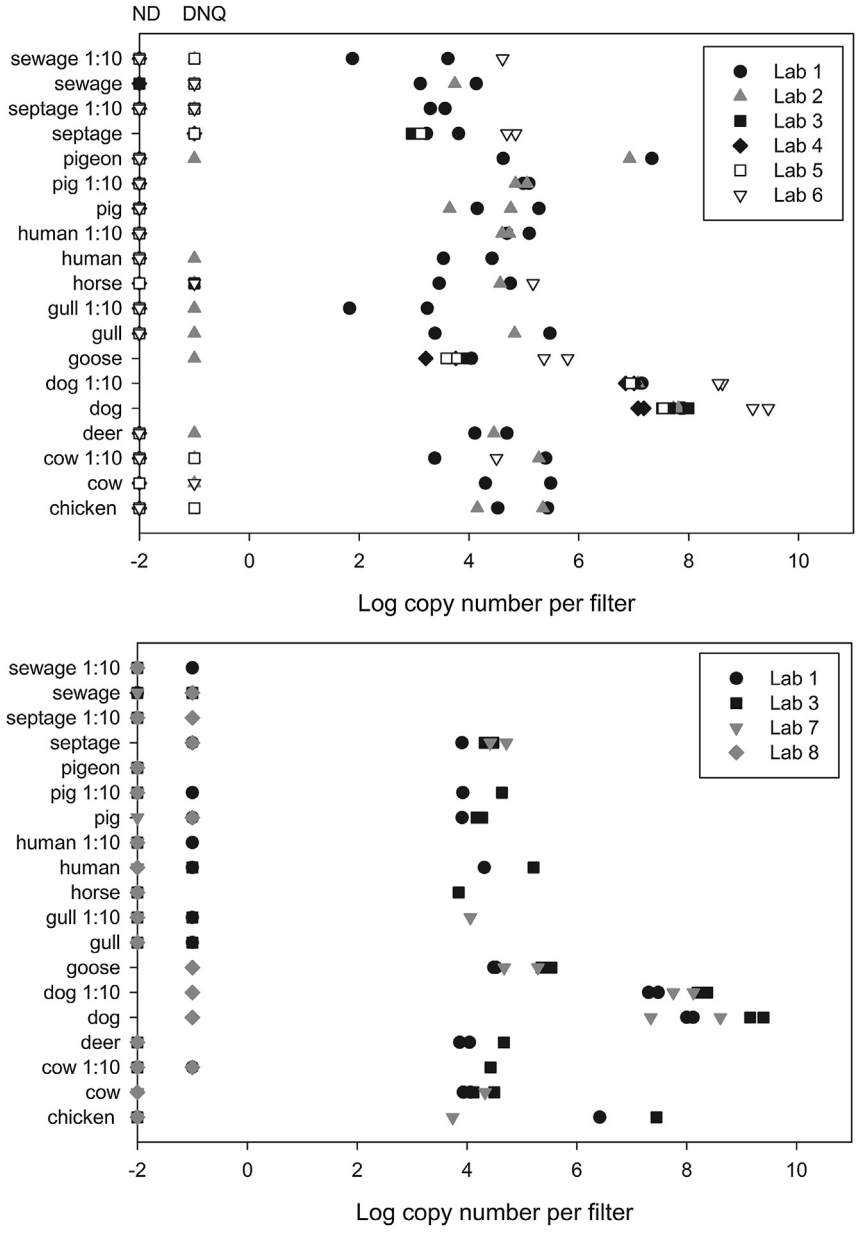

Fig. 2 – Results from 38 single-source (duplicate) samples for DogBact (top) and BacCan-UCD (bottom) after standardized data treatment. ND = not detected. DNQ = detected but not quantifiable.

divided by the total number of samples that did not contain the host feces:

$$\text{Specificity} = \frac{TN}{(TP + FP)} \quad (2)$$

where TN and FP are true negatives and false positives, respectively.

2.5. *Normalization of results*

Concentrations of DNA, General *Bacteroidales* (GenBac3), and viable enterococci provided by the research group that created the blinded sample set were used to normalize the results obtained from individual lab analyses for DogBact and BacCan-UCD assays. We also developed a new approach based on dividing measured gene copies by the relative standard deviation (RSD) of all analyses to account for inter-laboratory variation. Cultivable enterococci were measured by membrane filtration according to EPA method 1600 (US-EPA, 2006). Between two and three dilutions were filtered for enumeration. If more than one dilution was countable (between 1 to 250 CFU per filter), then the counts were averaged to estimate CFU per 100 mL. The concentration of GenBac3 was estimated by three labs using EPA Method B (US-EPA, 2010), with a geometric mean of values among laboratories calculated to estimate cp per 100 mL. The concentration of DNA was estimated by two laboratories using Quant-iT kits (dsDNA High-Sensitivity or dsDNA Broad-Range; Invitrogen-Molecular Probes, Grand Island, NY) that were applied to 50 mL of slurry filtered through a membrane filter and using

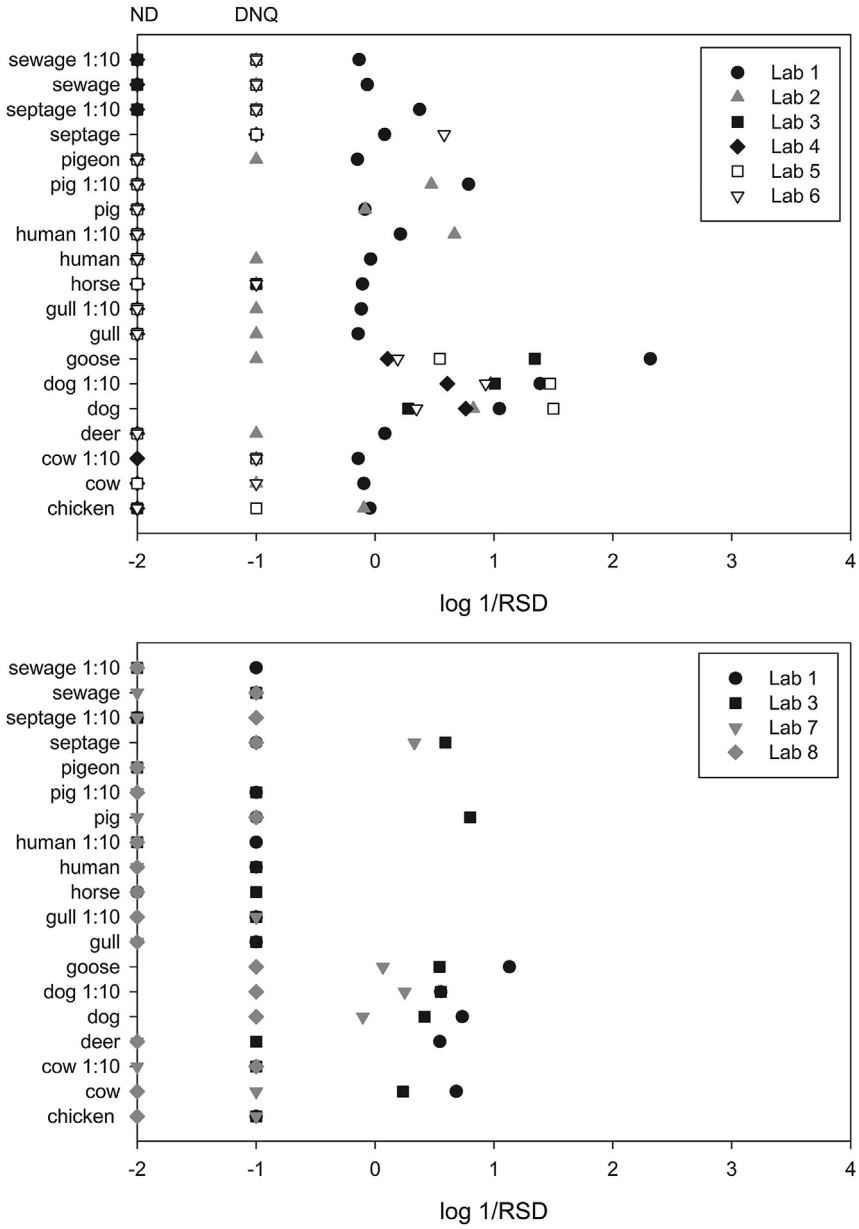

Fig. 3 – Presentation of DogBact (top) and BacCan-UCD (bottom) results as 1/RSD. Results of post standardized data treatment from 38 single-source (duplicates) samples are used. ND = not detected. DNQ = detected but not quantifiable (assigned when either of the two replicate samples was DNQ or one ND and the other detected).

GeneRite DNA extraction kits. This concentration was multiplied by the exact volume of extract and normalized to 100 mL of slurry. For detailed information see Boehm et al. (2013) and Supplementary Data.

3. Results

The DogBact (6 laboratories) and BacCan-UCD (4 laboratories) assays for the detection of canine associated *Bacteroidales* were tested on 64 challenge samples comprising duplicates of 19 singleton and 13 doubleton samples, prepared from pools of individual fecal samples collected across California. Several laboratories tested both qPCR assays. In general, laboratories were not restricted in how they processed samples or calculated and reported their results. This non-restriction led to two obvious differences among the laboratories: 1) the number of replicates and dilutions for samples and standard curves run by each lab and 2) the classification of results into ROQ, DNQ and ND. While the first difference influenced the quality of quantitative data, the second one directly affected qualitative outcomes, i.e., the assessment whether a sample was considered positive or negative (Table 2). To unify these outcomes it was necessary to treat all laboratories' original data in a standardized fashion. More specifically, the same criteria were used for the calculation of standard curves, which affected how outliers within the standard curve datasets were identified and LLOQs were calculated. After obtaining the standardized standard curves the same criteria were applied to the individual lab-specific datasets for the

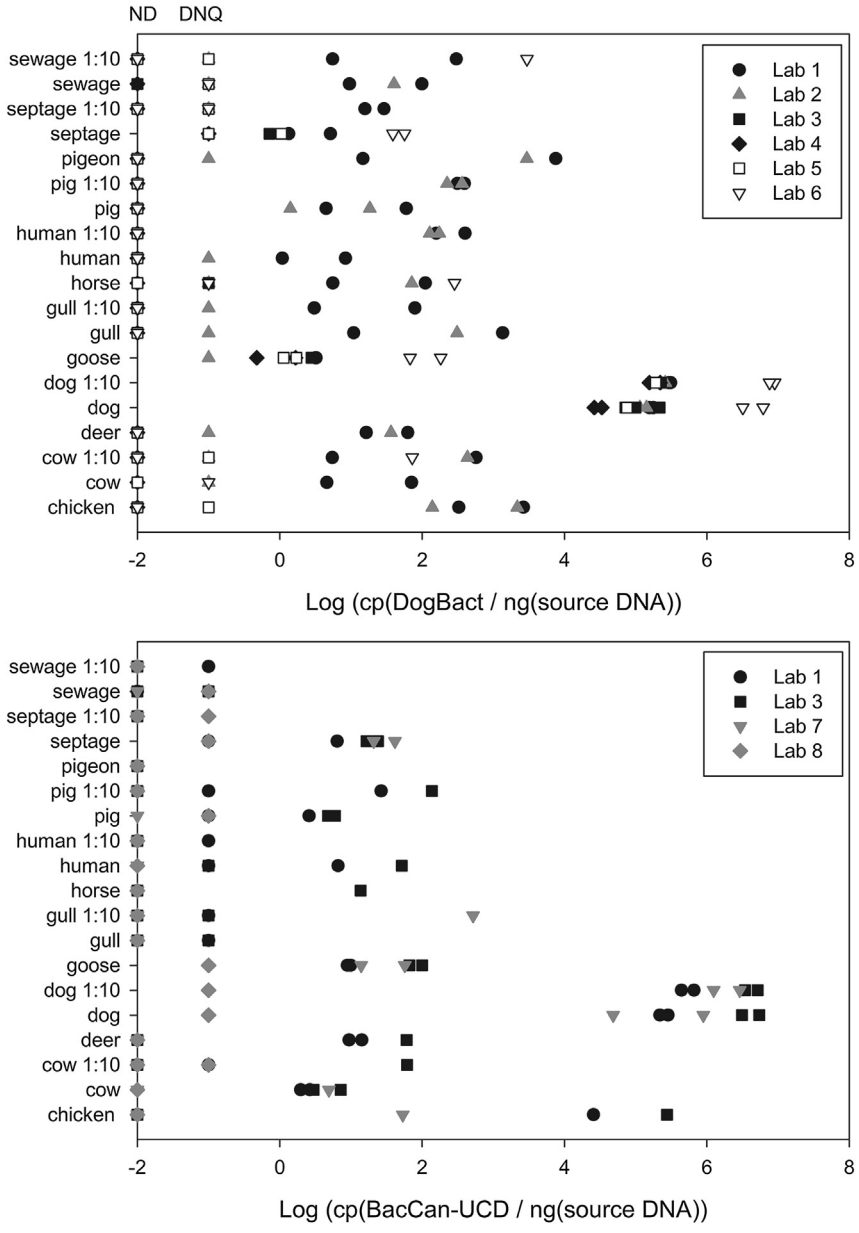

Fig. 4 – Normalization of DogBact (top) and BacCan-UCD (bottom) results by ng DNA measured in the sources. Results of post standardized data treatment from 38 single-source (duplicates) samples are used. ND = not detected. DNQ = detected but not quantifiable.

challenge samples to classify ROQ, DNQ, and ND consistently. Because the standardized data treatment resulted in close to zero specificity or sensitivity for data from lab 1 (DogBact) and lab 8 (BacCan-UCD), respectively, their data were excluded from the calculation of average sensitivities and specificities (Table 2). Both datasets represent special cases in which most ROQs had to be classified as DNQ or vice versa after application of standardized criteria.

3.1. *Effect of standardized data treatment on assay sensitivity and specificity*

Samples with an assay response below concentrations of the calculated laboratory's LLOQ were considered DNQ. Consideration of DNQ samples as positive samples resulted without exception in lower average specificity of assays without affecting sensitivities (Table 1 and Table S5 in Supplementary data). The calculated average specificity of the DogBact assay increased significantly after standardized data treatment from 0.46 to 0.81 when all samples were considered and from 0.49 to 0.83 when only singletons were considered. In contrast, the differences in calculated average specificity for the BacCan-UCD assay before and after standardization of results were only minimal with changes of 0.68–0.70 for all samples and 0.70 to 0.69 for singletons only. Besides concentrated and diluted dog singleton challenge samples, goose and septage samples were also reported by the majority of laboratories as above DNQ for both assays, and cow and chicken for only the BacCan-UCD assay. Fecal samples from dogs were collected in urban areas, minimizing the

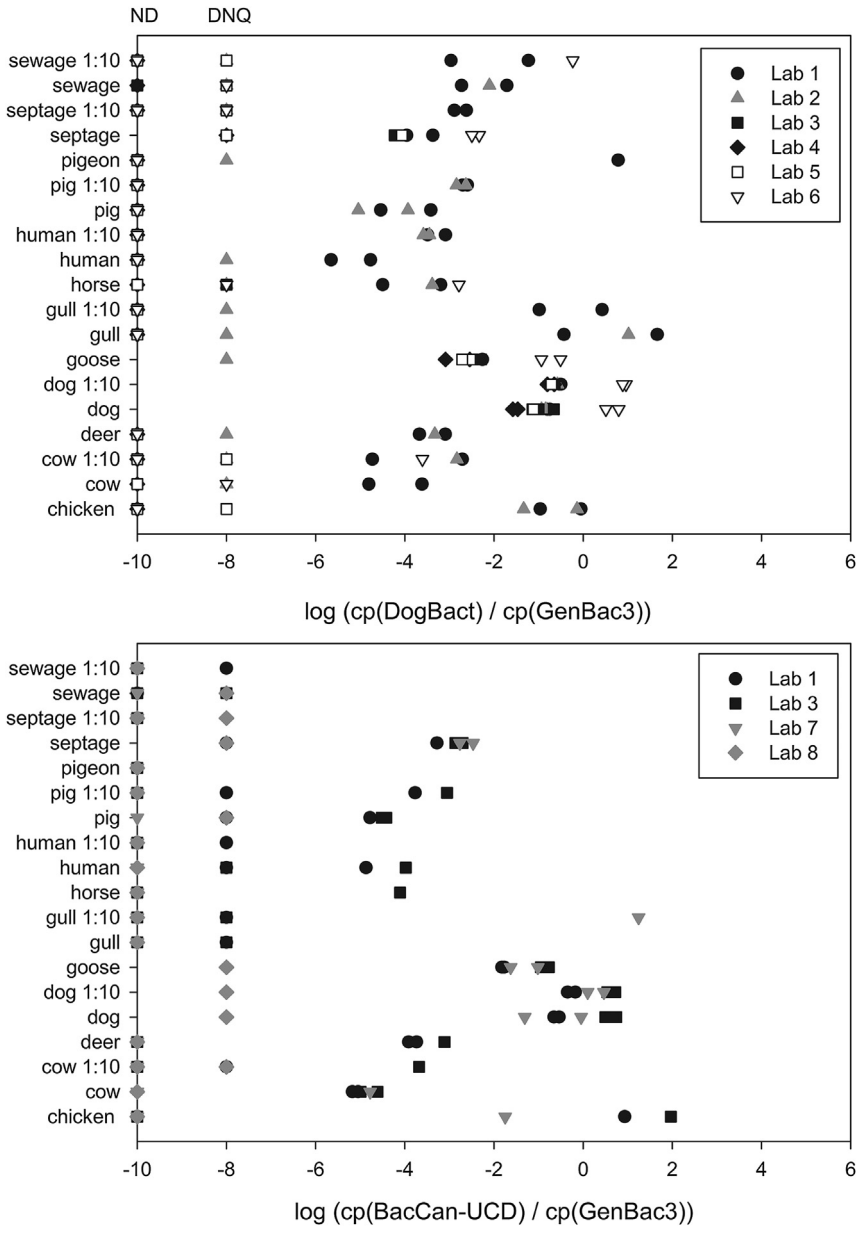

Fig. 5 – Normalization of DogBact (top) and BacCan-UCD (bottom) results by GenBac3 copy numbers. Results of post standardized data treatment from 38 single-source (duplicates) samples are used. ND = not detected. DNQ = detected but not quantifiable.

probability of direct contact between chicken and dogs. The *Bacteroidales* population in chicken appears to be rather diverse compared to other animal hosts and similar results were observed for other non-dog markers (unpublished information).

As expected, the highest signals were reported for both assays for the dog singleton samples, but pigeon (DogBact) and chicken (BacCan-UCD) singleton samples exhibited similar concentrations among some of the labs (Fig. 1; Fig. 2). Interestingly, for the DogBact assay at least one replicate filter from every sample was reported in the quantifiable range (ROQ) by at least one of the laboratories. This was also the case for the BacCan-UCD marker with the exception of gull, pigeon, and diluted sewage and human samples. Although standardized data treatment removed false positive sewage and diluted septage responses among the BacCan-UCD results by re-classifying the ROQ values to DNQ, most other non-target samples were measured clearly above LLOQ values. These results, particularly the differences observed between replicate filters, motivated additional data analysis.

3.2. *Effect of different normalization procedures on sensitivity and specificity of assays*

What distinguishes qPCR from PCR – as the name suggests – is the ability to generate quantitative data, which allows data conversion beyond binary presence/absence observations. The following section describes methods to normalize the dog-associated *Bacteroidales* concentration data to ideally identify non-target responses (false positives).

3.2.1. *Consideration of relative standard deviation*

If the observed non-target responses were due to random effects, like qPCR signal artifacts caused by irregularities in reaction mixture or material, possible cross contamination, or sample mix-up, it would be unlikely for them to appear in both duplicates of the same sample to the same extent. True positives on the other hand should deliver an intense (that is, high concentration) and reproducible response. As a way to eliminate positives due to such errors ('random positives'), we used the relative standard deviation between the two replicates. By displaying the reciprocal 1/RSD values, true positives should be found towards the right side of the diagram. This treatment led to a significant reduction of non-target data points; yet, chicken, human and pig samples remained above DNQ criteria reported by at least two laboratories for the DogBact assay, and cow and septage samples remained above DNQ criteria for BacCan-UCD (Fig. 3). For both assays, goose and dog samples were the only samples for which data points from the majority of laboratories remained positive. Taking into account that these standard deviations comprised all possible sources of variability, from sample filtration to sample extraction and quantification, we considered an RSD of 150% as still reasonable and thus applied a threshold value of log $(1/RSD_{150}) = -0.176$ for sensitivity and specificity calculations (Table 2(d)). The resulting average specificities were 0.89 and 0.82 for DogBact and BacCan-UCD assays, respectively. As expected, the sensitivity was not affected by this data conversion and remained at 1.00.

3.2.2. *Normalization of quantitative qPCR data by total DNA concentration*

The total DNA concentration of a sample is a good indication of its total biomass. This particular study used a defined sample set of known feces; therefore, a correlation between target concentration and total DNA was expected (barring random variation and possible cross-contamination, as discussed above). Thus, normalizing qPCR concentrations by measured total DNA concentrations represented another way to reduce random non-target signals. This normalization (log (copies assay/ng source DNA)) lead to a clear separation of dog singleton samples among all laboratories. For DogBact, the target samples were separated from the other samples at a value greater than four log gene copies per nanogram DNA on the *x*-axis (Fig. 4). For the BacCan-UCD assay, this same clustering above four on the *x*-axis (log scale) was seen for target samples, with the exception that cross-reactivity with chicken samples was observed by two labs, with results reported in the same range as dog singleton samples (Fig. 4). It is unclear as to why a strong separation occurred at this particular value, but nonetheless a set of samples clustered at about four magnitudes higher than other samples. This threshold appeared to separate samples that gave true positive signals because when this observational threshold was used to classify data in a binary analysis, the average DogBact and BacCan-UCD specificities became 1.00 and 0.98, respectively. Again, the sensitivities of 1.00 for both assays were not affected by the normalization.

3.2.3. *Normalization of quantitative qPCR data by GenBac3 concentration*

Many of the MST markers used in the SIPP study targeted source-associated *Bacteroidales*, in part because this order is so abundant in a variety of feces. Thus, normalizing host-associated *Bacteroidales* qPCR data against non-host-associated (universal) *Bacteroidales* qPCR data (GenBac3 results) may provide improved normalization compared to other methods (e.g., versus normalization to enterococci). Normalization to GenBac3 did indeed lead to enhanced cluster formation of data points for the BacCan-UCD assay (Fig. 5). In contrast, DogBact results were mostly unaffected.

Using the assumption that a host-associated marker would need to represent at least 1% of the universal *Bacteroidales* marker concentration to be of utility in real-world samples, gives a ratio of dog-associated *Bacteroidales* to general *Bacteroidales* of 0.01 (i.e., $\text{Log}(0.01) = -2$) as a conservative binary threshold for calculation of sensitivities and specificities. This analysis resulted in average specificities of 0.96 and 0.90 for DogBact and BacCan-UCD assays, respectively. Again, the average sensitivities remained at 1.00 for both assays.

4. Discussion

4.1. *Determining most probable cross-reactivity*

Limited information about cross reactivity with non-target fecal samples for the two dog-associated *Bacteroidales* markers used in this study was available. In designing the probe, Sinigalliano et al. (2010) converted a published dog-associated *Bacteroidales* conventional PCR assay (Dick et al., 2005a) to the

DogBact qPCR assay used in this study (Sinigalliano et al., 2010). The SIPP study is the first to report cross-reactivity data for this qPCR assay (additional specificity testing data are given in supplementary data, see also Boehm et al., 2013). Kildare et al. (2007) previously reported cross-reactivity of BacCan-UCD with individual human (4/18), pooled cat (1/7), and wastewater influent (4/14) samples. Cross-reactivity also occurred with pooled cow (11/11), horse (2/10), and goose (5/10) fecal samples as well as sewage (10/12) (Silkie and Nelson, 2009). Other studies modified the BacCan-UCD assay significantly, either by using only conventional PCR and removing the second reverse primer (Ahmed et al., 2008) or by choosing a different annealing temperature (Tambalo et al., 2012b), and thus cannot be used for a valid comparison of reported cross-reactivity of the assay.

The standardized data treatment removed variations among laboratory results due to data processing. Comparing the standardized datasets for singleton challenge samples of participating laboratories confirmed that some cross reactivity was observed for both assays for goose and septage, and also for chicken for BacCan-UCD. However, other cross-reactivities were not consistent between sample replicates or across laboratories, indicating random errors perhaps introduced by cross-contamination, qPCR artifacts, or other unidentified reasons. Differences in detection limits of the different laboratories could also have been a factor for low level cross-reactivity as the reported LLOQ values varied significantly among laboratories (Table 1).

Consequently, reports of false positives with non-targets must be viewed with caution when observed in a minority of non-target samples or by only one laboratory, whether from individual or composite samples.

4.2. *Consequences for MST studies*

Statistical models can help to adjust host distributions relative to each other by translating measured concentrations into probable distributions by accounting for uncertainties and errors (Wang et al., 2010). The accuracy of such treatment strongly depends on the strength of the original validation of assay specificity and sensitivity. However, not all assays used in the SIPP study were validated in a similar fashion, and this may have confounded assay comparisons. Many papers in the SIPP study presented differential performance characteristics for assays depending on how qPCR data were binned into binary results, with particular focus on treatment of DNQ results (Layton et al., 2013, Raith et al., 2013, Sinigalliano et al., 2013). Validation requires adequate replication (Armbruster and Pry, 2008; CODEX, 2010), and variations in LOD and LLOQ calculations (Stewart et al., 2013) can directly affect how quantitative results are binned for binary analysis. It is likely that if all assays had been similarly and rigorously validated, creating accurate limit of detection and limit of quantification statistics, much of the need for interpretation would have disappeared. Clearly, the overall SIPP study has highlighted the necessity of uniform protocols to implement MST across laboratories (Ebentier et al., 2013). In addition, the results in total suggest a requirement for defined assay validation procedures prior to moving assays into inter-laboratory verification exercises. Validation needs to include definition of matrix effects, and for MST applications, sample aging. Genotyping or metagenomic sequencing of samples (Rosario et al., 2009; Tringe and Rubin, 2005) would also benefit future validation efforts.

In the absence of additional validation exercises, any type of positive MST results will be subject to uncertainty, whether the observed signal is a result of a small load of real host feces or due to a larger load of cross-reactive non-host feces. One possible solution is normalization by other related parameters, assuming that the ratio of true associated host signal to, e.g., total DNA would be much bigger than if the reason for the signal were the presence of much more (cross-reactive) DNA material. While measurements like *Enterococcus* and total DNA concentrations are relatively simple to perform, they may not be related to host-associated *Bacteroidales* data. Normalization by total DNA concentration was effective in separating target from non-target samples in this study. This result was likely due to the fact that the samples contained only mixed fecal material so that total DNA concentration was an adequate proxy for the DNA concentration of the fecal bacteria predominant in the sample; this relationship is unlikely to hold with real environmental samples. In contrast, normalization by *Enterococcus* did not aid assay performance (Table S1, supplementary data). Notably, the ratio of enterococci to each MST marker is expected to vary. The *Enterococcus* population can vary significantly in feces of different animals and in relation to *Bacteroidales* and hence may not be optimal for normalization (Silkie and Nelson, 2009; Ervin et al., 2013).

Normalization of these *Bacteroidales*-based MST markers to general *Bacteroidales* may provide a better method of normalization. Correlations between host associated and general *Bacteroidales* concentrations have been observed (Schriewer et al., 2010; Silkie and Nelson, 2009), although variations for individual fecal samples do occur. In this study, normalization of host-associated *Bacteroidales* (DogBact and BacCan-UCD) assays to general *Bacteroidales* (GenBac3 assay) improved assay performance. Elimination of samples with less than 1% of host-assay contribution towards the general *Bacteroidales* concentration as non-target resulted in an improvement of specificity from 0.83 to 0.96 for the DogBact assay and from 0.69 to 0.90 for the BacCan-UCD assay after standardization of data treatment. The normalization of host-associated *Bacteroidales* with general *Bacteroidales* marker results for environmental samples seems plausible, but criteria for the identification of main sources need to be carefully considered based on the assays tested.

5. Conclusions

Two MST assays for the detection of dog-associated *Bacteroidales* were tested by six (DogBact) and four (BacCan) laboratories against 64 created single-source and mixed-source samples from pooled individual fecal samples collected across California.

- Assay sensitivity and specificity depended on the way data analysis was performed. Standardized data treatment of standard curve and sample data regarding outliers, lower limit of quantification (LLOQ), non-detects (ND), and detected but

not quantifiable (DNQ) definitions, proved indispensable for consistent determination of assay sensitivity and specificity.
- In this study, random non-target responses needed to be separated from consistent 'real' non-target cross-reactivity of assays.
- Normalization of host-target *Bacteroidales* data by general *Bacteroidales* results (GenBac3 assay) helped eliminate apparent cross-reactivity.
- The ability to treat data beyond binary presence-absence observations was a marked advantage of qPCR compared to PCR for assay validation studies.
- Both assays exhibited 100% average sensitivity with all presented normalization and non-normalization variations. After standardized treatment the average specificity was 83% and 68%, for the DogBact and the BacCan-UCD assays, respectively. Normalization by general *Bacteroidales* concentrations yielded 96% and 90% average specificity for DogBact and BacCan-UCD, respectively, when dog-associated *Bacteroidales* proportions of less than one percent were considered non-detect.
- Overall, given the variability of results among the eight laboratories it can be concluded that both assays performed equally well in this study.

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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.2013.03.062.

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