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Swine-associated microbial source-tracking markers in environmental water: a systematic review of diagnostic performance and field application

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Abstract

Swine-associated fecal contamination can impair environmental-water quality and contribute to the transport of zoonotic pathogens and antimicrobial-resistant organisms. We systematically reviewed the performance and environmental application of four swine-associated microbial source-tracking markers: Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2. Searches across six databases identified 911 records; 34 primary studies of environmental-water matrices were included following PRISMA 2020. Pig-2-Bac had the broadest field-application record, but its reported diagnostic sensitivity varied across validation studies and sample collections. Controlled aquatic-microcosm persistence data have been reported for both Pig-1-Bac and Pig-2-Bac, with results dependent on experimental conditions and the endpoint assessed, including concentration reduction, quantification, and residual detection. Evidence for Pig-1-Bac was more limited but generally indicated high performance in the host collections evaluated, whereas evidence for Pig-Bac-2 was sparse. P23-2 showed variable and lower fecal-detection estimates across independent evaluations, with distinct outcome definitions, but may provide complementary evidence because it targets an archaeal gene. Pig-2-Bac is the best-supported candidate for initial monitoring of swine-associated fecal contamination in environmental water; however, local validation against representative swine and non-swine reference collections remains essential, particularly in tropical karst groundwater and cenote systems.

1. Introduction

Fecal contamination of environmental waters is a persistent public health and environmental-management challenge. Conventional fecal indicator bacteria (FIB), including total coliforms, fecal coliforms, and Escherichia coli, have long been used to indicate fecal pollution in water-quality assessment. Although FIB can indicate the presence of fecal contamination, they generally do not identify its host source—human, livestock, or wildlife—thereby limiting targeted remediation, exposure assessment, and regulatory action in watersheds affected by multiple fecal inputs (Hagedorn et al., 2011; Harwood et al., 2014). In environmental monitoring, source-specific molecular markers can complement conventional fecal indicators by linking microbial contamination to likely host sources and thereby supporting targeted mitigation of agricultural pollution.

This limitation is especially relevant in agricultural regions, where human, livestock, domestic animal, and wildlife sources may co-occur. Swine-associated fecal contamination is of particular concern because it may introduce zoonotic pathogens and antimicrobial-resistant bacteria into surface water and groundwater. Relevant hazards include Salmonella spp., Yersinia enterocolitica, Cryptosporidium spp., hepatitis E virus, and antimicrobial-resistant bacteria, although occurrence and risk vary by production system, environmental conditions, and local epidemiology (Mataragas et al., 2008; Van Boeckel et al., 2019). Beyond aquatic environments, Kurowski et al. (2025) found that greater swine production intensity was associated with more frequent detection and higher quantities of Pig-2-Bac DNA in settled dust on household surfaces in North Carolina, USA, providing evidence of swine-associated fecal contamination in residential environments. Schiaffino et al. (2020) also evaluated Pig-2-Bac among eight

MST markers for attributing fecal contamination in indoor-household environments of the Peruvian Amazon.

Microbial source tracking (MST) provides a framework for attributing fecal contamination in environmental waters to particular host sources. Among MST approaches, molecular assays targeting host-associated microorganisms have been widely applied because they can be detected by conventional PCR, quantitative PCR (qPCR), or digital PCR (dPCR) and may distinguish among fecal sources that cannot be differentiated by conventional FIB monitoring alone (Kildare et al., 2007; Reischer et al., 2011). Bacteroidales-based markers are commonly used in MST because members of this order are abundant in intestinal microbiota and are generally anaerobic, which limits their environmental persistence and proliferation relative to many conventional bacterial indicators. Their detection in water can therefore provide evidence consistent with fecal input. However, marker performance depends on the assay, host reference collection, geography, environmental matrix, DNA extraction approach, PCR platform, and analytical threshold. Consequently, marker performance should be evaluated in the setting in which it will be applied. A meta-analysis of 46 studies further identified methodological and geographical factors as potential influences on MST marker performance, reinforcing the need for context-specific interpretation of published estimates (Liu et al., 2023).

The development of molecular assays for swine-associated MST has been motivated by intensive swine production systems, which generate large volumes of manure and can affect surface water and groundwater through runoff, leaching, land application, lagoon overflow, and other transport pathways (Ufnar et al., 2007; Heaney et al., 2015). Four candidate markers have been repeatedly evaluated for tracking swine-associated fecal contamination in environmental water. Pig-1-Bac and Pig-2-Bac target swine-associated Bacteroidales 16S rRNA sequences and were originally developed as TaqMan qPCR assays (Mieszkin et al., 2009). Pig-Bac-2 was evaluated alongside Pig-1-Bac, Pig-2-Bac, and P23-2 by Heaney et al. (2015). P23-2 targets the archaeal mcrA gene associated with methanogenic microorganisms in swine fecal material and was originally described using conventional PCR (Ufnar et al., 2007). These assays differ in biological target, format, available validation data, and extent of field application. Other swine-associated molecular targets have also been investigated. Duan et al. (2016) developed the PFB-1 and PFB-2 qPCR assays targeting swine-associated *Faecalibacterium* 16S rRNA gene sequences for detecting fecal contamination in water. Fan et al. (2020) also evaluated alternative swine-associated Bacteroidales markers, designated gene 1–38 and gene 3–53, using qPCR in water samples from the Yangtze Delta watershed, China.These assays represent alternative targets beyond the four candidate markers selected for this review.

More than 15 years after the original studies, the literature includes analytical validations, field applications, persistence experiments, and geographically distinct evaluations. However, these studies differ substantially in host reference collections, target and non-target sample numbers, environmental matrices, PCR platforms, reporting thresholds, and outcome definitions. Sensitivity, specificity, predictive values, area-under-the-curve metrics, environmental detection frequencies, and concentrations are therefore not interchangeable measures of performance.

Selection of a molecular marker for swine-associated fecal source tracking requires comparison of the available evidence across analytical performance, cross-amplification, environmental-water detection, persistence, and geographic applicability. Existing MST reviews have addressed broad classes of host-associated markers but have not provided a systematic, marker-by-marker synthesis of Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2 specifically for environmental-water matrices (Harwood et al., 2014; Reischer et al., 2011). This gap is particularly relevant in tropical, subtropical, and karstic regions, where rapid hydrological connectivity and groundwater vulnerability may increase exposure to fecal contamination, while local assay-validation data remain limited. A systematic synthesis can identify where evidence is strongest, where findings are context dependent, and what validation is required before routine implementation.

The objective of this systematic review was to identify and descriptively synthesize primary studies evaluating the analytical performance and field application of four swine-associated molecular markers—Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2—for fecal source tracking in environmental-water matrices. We compared evidence on analytical sensitivity and specificity, cross-amplification, field detection, aquatic persistence, water-matrix applicability, and geographic scope of evaluation. Because host reference collections, assay formats, matrices, and performance definitions were heterogeneous, no formal pooling of diagnostic-performance estimates was planned.

2. Materials and methods

2.1 Search strategy

A systematic literature search was conducted using six complementary sources to maximize coverage of the published literature. Database and discovery-source searches were completed on September 30, 2026, without language restrictions. Search results from all six sources were exported, merged, managed in Mendeley reference management software, and deduplicated before title and abstract screening. Elicit (elicit.com), an artificial intelligence-assisted evidence-synthesis platform that retrieves and structures literature from indexed sources, including Semantic Scholar, was searched using seven independent queries: “Pig-1-Bac”; “Pig-2-Bac”; “P23-2 porcine fecal marker”; “Pig-Bac-2 porcine fecal marker”; “swine-associated Bacteroidales qPCR water validation”; “swine fecal source tracking marker comparison”; and a comparative query combining P23-2, Pig-1-Bac, and Pig-2-Bac. PubMed (National Library of Medicine) was searched using three independent queries: (1) “Pig-1-Bac OR Pig-2-Bac OR Pig-Bac-2 OR P23-2 AND fecal AND water”; (2) “swine fecal source tracking Bacteroidales water qPCR”; and (3) “swine-associated molecular marker environmental water microbial source tracking”. Dimensions (app.dimensions.ai) was searched using two queries, with field-of-research filters for Biological Sciences, Microbiology, Environmental Sciences, and Health Sciences: (1) “Pig-2-Bac” OR “Pig-1-Bac” OR “Pig-Bac-2”; and (2) “swine fecal Bacteroidales water qPCR source tracking”. Google Scholar was searched using: (1) “Pig-2-Bac OR Pig-1-Bac swine fecal water”; and (2) “P23-2 OR Pig-Bac-2 swine fecal marker water PCR”. Search was limited to publications from 2007 to 2026. ScienceDirect (Elsevier) was searched with subject-area filters for Environmental Science and Microbiology using four queries: (1)

“Pig-2-Bac” OR “Pig-1-Bac” OR “Pig-Bac-2” OR “P23-2” AND swine AND water; (2) “swine fecal Bacteroidales water qPCR source tracking”; (3) “microbial source tracking swine molecular marker environmental water”; and (4) “Pig-2-Bac” OR “Pig-1-Bac” OR “Pig-Bac-2” OR “P23-2” fecal marker water. Web of Science Core Collection was searched using three queries: (1) TS=(“Pig-2-Bac” OR “Pig-1-Bac” OR “Pig-Bac-2” OR “P23-2”) AND TS=(swine OR pig) AND TS=(water OR fecal); (2) TS=(“swine fecal” AND Bacteroidales AND water AND qPCR); and (3) TS=(“microbial source tracking” AND swine AND “molecular marker” AND water). Searches were limited to publications from 2007 to 2026 and document types classified as articles or reviews.

Backward and forward citation searching was conducted for all included primary studies to identify additional potentially eligible reports. The complete search strategies, including exact search strings, search fields, filters, search dates, and numbers of records retrieved for each query, are provided in Supplementary Table 1. Elicit and Google Scholar were used as supplementary discovery tools; their search outputs may be less reproducible than those of conventional bibliographic databases. ScienceDirect was used as an additional source but predominantly indexes Elsevier-published content. Scopus was not searched because institutional access was unavailable; the potential implications of this limitation are addressed in the Discussion.

2.2 Eligibility criteria

Primary studies were eligible if they: (1) evaluated the design, analytical validation, or field application of at least one candidate swine-associated marker—Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, or P23-2; (2) included an environmental-water matrix, defined as surface water, groundwater, spring water, runoff, coastal water, recreational water, drinking-water sources, wastewater, or aquatic microcosms; (3) used a PCR-based method, including conventional PCR, quantitative PCR (qPCR), or digital PCR (dPCR); and (4) reported marker-specific analytical performance, field detection, concentration, persistence, or cross-amplification data independently from other host-associated markers.

Studies were excluded if they: (1) were clinical, veterinary, occupational, household-surface, fecal-only, soil-only, or sediment-only studies without an environmental-water component; (2) did not evaluate at least one of the four candidate markers; (3) used only non-PCR-based methods; or (4) were conference abstracts, theses, book chapters, non-peer-reviewed preprints, narrative reviews, or other non-eligible document types; or (5) had an insufficiently verifiable bibliographic record that precluded reliable data extraction after full-text retrieval and independent verification.

2.3 Study selection

Title and abstract screening and full-text assessment were initially performed by one reviewer (MG-K) and documented against the prespecified eligibility criteria. To strengthen the reliability of the final inclusion decisions, a second reviewer (MJS-S) independently reassessed the eligibility of all full-text reports, including both included studies and reports excluded after full-text review. Discrepancies were resolved through discussion and consensus. The selection process was reported using the PRISMA 2020 flow diagram (Fig. 1).

The protocol was registered retrospectively in OSF Registries (osf.io/rx3y5) on September 30, 2026, after data extraction had begun. The registered protocol documented the initial search strategy. Search-strategy amendments, including the addition of Web of Science and citation searching, as well as the subsequent independent verification of full-text eligibility, data extraction, and descriptive appraisal by a second reviewer, are documented in the OSF record and Supplementary Table 1.

2.4 Data extraction and synthesis

Data were initially extracted by one reviewer (MG-K) from each eligible primary study. A second reviewer (MJS-S) independently verified the completeness and accuracy of the extracted data against the original reports. Discrepancies were resolved through discussion and consensus.

Extracted variables included publication year, country, marker, biological target, assay format, host reference collection, water matrix, analytical sensitivity, analytical specificity, positive predictive value (PPV), negative predictive value (NPV), area under the receiver-operating-characteristic curve (AUC) or other classification metrics, cross-amplification, environmental detection frequency, concentration, persistence, and geographic context.

Sensitivity and specificity were extracted only when explicitly reported or directly calculable from published data with identifiable reference-sample denominators. Sensitivity was evaluated in known swine-source samples, whereas specificity was evaluated in known non-swine-source samples. Reference-collection composition was recorded to distinguish individual fecal samples, composite samples, and mixed source-sample collections. PPV, NPV, AUC, environmental detection frequency, concentration, and downstream-to-upstream prevalence ratios were recorded as separate study-specific outcomes and were not treated as sensitivity or specificity. Because host reference collections, assay formats, analytical thresholds, water matrices, and outcome definitions were heterogeneous, no formal meta-analysis or pooled estimate was performed. Extractable sensitivity and specificity estimates were reported descriptively as study-level results, with attention to reference-collection composition and assay conditions.

Reporting completeness and methodological transparency were appraised descriptively for each included primary study, considering study design, host reference-collection characteristics, analytical methods and controls, type of performance metric reported, and applicability to environmental-water monitoring (Supplementary Table 3). The appraisal was conducted by MG-K and independently verified by MJS-S. Discrepancies were resolved through discussion and consensus. Studies were classified as reported, partially reported, not reported, or not applicable for each domain. No formal risk-of-bias instrument was applied because the included studies comprised heterogeneous analytical-validation, field-application, persistence, and methodological-comparison designs.

3. Results

3.1 Study selection

Searches across six sources retrieved 911 records. After removal of 456 duplicates, 455 unique records were screened by title and abstract. Of these, 419 records were excluded because they were outside the scope of swine-associated microbial source tracking in environmental water (n = 186), evaluated non-candidate markers (n = 93), were non-eligible document types (n = 88), or used non-PCR methods (n = 52). Thirty-six full-text reports were sought and all were successfully retrieved. Four reports were excluded after full-text assessment: one did not include an environmental-water matrix, two were conference publications, and one had an insufficiently verifiable bibliographic record. The initial selection yielded 32 primary studies. Subsequent eligibility reassessment added Marti et al. (2011) and reclassified He et al. (2016) as an included primary study, bringing the final synthesis to 34 studies. He et al. (2016) was identified through post-selection citation verification of an included study and was not retrieved through the original database searches; it was therefore entered through the other-methods pathway of the PRISMA flow diagram.

3.2 Characteristics of included studies

The 34 included primary studies were published between 2007 and 2026 and evaluated one or more of the four candidate markers—Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2—in environmental-water matrices. These matrices included surface water, groundwater, karst springs, agricultural runoff, recreational water, drinking-water sources, coastal water, and aquatic microcosms. The studies represented multiple countries across five continents. Conventional PCR and qPCR were the most frequently used assay formats, and one study applied digital PCR. Characteristics of the included primary studies are summarized in Supplementary Table 1.

Reporting completeness and methodological transparency varied across studies and are summarized in Supplementary Table 3. Analytical validation studies more commonly reported host reference-collection composition and directly defined sensitivity and specificity, whereas field-application and persistence studies generally reported environmental detection, concentration, or persistence outcomes rather than diagnostic-performance metrics.

3.3 Marker-specific findings

3.3.1 Pig-1-Bac

Pig-1-Bac is a swine-associated molecular marker targeting a Bacteroidales 16S rRNA gene sequence. It was originally developed and evaluated by Mieszkin et al. (2009) using a TaqMan qPCR assay and a reference collection of swine and non-swine fecal samples. In that study, Pig-1-Bac showed 100% specificity (54/54 non-swine fecal samples negative) and 100% sensitivity in the 25 individual swine fecal samples; across the mixed collection of 69 swine-source samples (25 fecal, 23 slurry, 14 lagoon-water, and 7 compost samples), sensitivity was 98.6% (68/69), calculated from the original Table 2. Among eight river-water samples collected near pig farms in the Daoulas River catchment in France, Pig-1-Bac was quantified in 25% of samples (3.9–4.0 log10 gene copies per 100 mL) and detected in 37.5%.

Heaney et al. (2015) reported 80% sensitivity for Pig-1-Bac by TaqMan qPCR in a mixed collection of 15 swine-source samples (feces, wallow water, and waste-lagoon water) and 100% specificity in 19 non-swine fecal samples; sensitivity was 92% when the two negative wallow-water samples were excluded. In the same comparative field study near concentrated animal feeding operations in the United States, Heaney et al. (2015) reported Pig-1-Bac at 2.47 times greater prevalence in downstream than upstream water samples (95% CI: 1.03–5.94). Together, these studies indicate that Pig-1-Bac can perform well in the host collections evaluated, although its environmental field-application record was smaller than that identified for Pig-2-Bac.

3.3.2 Pig-2-Bac

Pig-2-Bac targets a swine-associated Bacteroidales 16S rRNA gene sequence and was co-developed with Pig-1-Bac by Mieszkin et al. (2009) using TaqMan qPCR. In the original validation, Pig-2-Bac showed 100% specificity (54/54 non-swine fecal samples negative) and 100% sensitivity in both the 25 individual swine fecal samples and the mixed collection of 69 swine-source samples (69/69). Among the same eight river-water samples, Pig-2-Bac was quantified in 62.5% (3.6–4.1 log10 gene copies per 100 mL) and detected in 87.5%, compared with 25% and 37.5%, respectively, for Pig-1-Bac. Gourmelon et al. (2010) evaluated microbial and chemical source-tracking tools in wastewater, runoff, and river-water samples and selected Pig-2-Bac, together with *Lactobacillus amylovorus*, as swine-associated components of a multi-marker MST toolbox.

Pig-2-Bac was detected in runoff from swine-manure-amended plots but not from cattle-manure-amended plots under the conditions evaluated (Jaffrezic et al., 2011). In a two-year French catchment study, Mauffret et al. (2012) reported low baseline detection frequency (0–4 of 245 samples) and increased occurrence during manure-application events. In controlled aquatic microcosms maintained in darkness at 16–20°C, Solecki et al. (2011) reported Pig-2-Bac T90 values of 1.9 days in freshwater and 2.1 days in seawater. In freshwater, the marker remained above the quantification limit through day 27 and was still detectable at day 34 in two of three microcosms. In seawater, it reached the quantification limit around day 13, was below it by day 20, and remained detectable in two microcosms the following week. Additional controlled aquatic persistence data were reported by Marti et al. (2011), who investigated Pig-1-Bac and Pig-2-Bac in river water inoculated with pig manure at 4 and 20°C under aerobic and microaerophilic conditions. Exact T90 values from this experiment were not retained because of discrepancies between the published abstract and sections of the accepted manuscript. T90 represents a 90% reduction in marker concentration, not loss of detection, and these estimates should not be directly equated with the quantification and detection windows reported by Solecki et al. (2011). He et al. (2016) also investigated Pig-2-Bac decay in laboratory and field aquatic microcosms, demonstrating that temperature and light exposure influenced marker persistence.

Reported Pig-2-Bac sensitivity varied among studies and source collections. Although several studies reported 100% sensitivity, Vadde et al. (2019) reported 90% sensitivity in individual swine fecal samples. Heaney et al. (2015) reported 87% sensitivity in a mixed collection of 15 swine-source samples comprising feces, wallow water, and waste-lagoon water, and 100% specificity in 19 non-swine fecal

samples. The authors noted that sensitivity would have reached 100% if the two negative wallow-water samples had been excluded. These results should be interpreted in relation to the composition of each reference collection. For example, Gómez-Doñate et al. (2016) reported an AUC of 0.65 for Pig-2-Bac under study-specific conditions; this metric was not treated as a measure of specificity.

In a host reference collection from the Taihu Lake watershed, China, He et al. (2016) reported 100% sensitivity (25/25 swine fecal samples) and 100% specificity (107/107 non-swine fecal samples) for Pig-2-Bac using conventional PCR. The study also quantified Pig-2-Bac decay in laboratory and field aquatic microcosms using SYBR Green-based qPCR. In Thailand, Somnark et al. (2018) reported 100% sensitivity and 98–100% specificity across two watershed-specific host collections. PPV values of 85–100% and NPV values of 90–100% were also reported separately and were not interpreted as sensitivity or specificity. Malajacan et al. (2023) reported 100% sensitivity and 100% specificity in the host collection evaluated in the Philippines and detected Pig-2-Bac in Laguna Lake, with a mean concentration of 2.47 log10 copies/mL.

Several field applications further documented Pig-2-Bac in diverse environmental-water settings. McKee et al. (2021) applied Pig-2-Bac in recreational surface waters of Congaree National Park, South Carolina, USA, where it was the most frequently detected MST marker; the authors identified feral swine as the principal source of the swine-associated signal in the absence of hog farms within the watershed. Christenson et al. (2022) detected Pig-2-Bac at 3.5 times more sites downstream of commercial hog operations than at background sites ($p = 0.035$). Liang et al. (2021) identified Pig-2-Bac as the best-performing swine-associated marker among the candidate assays evaluated in a rural river in Beijing. Vadde et al. (2019) detected the marker in river water and sediments in the Tiaoxi River system; detection occurred in 64% of sediment samples, while water concentrations were reported separately. In the regional fecal reference collection, Vadde et al. (2019) reported Pig-2-Bac sensitivity of 90% (9/10 swine samples) and specificity of 95%, with cross-amplification in two cattle samples. Zhang et al. (2020) found that non-target amplification occurred at concentrations 1–3 orders of magnitude lower than target-sample concentrations in its large Chinese host collection.

Additional studies reported use or evaluation of Pig-2-Bac in groundwater, drinking-water sources, tropical rivers, coastal waters, and recreational waters. For example, Sekgobela et al. (2023) detected Pig-2-Bac in 15.7% of rural boreholes during the wet season in South Africa. Mudau et al. (2023) also applied Pig-2-Bac, alongside human-, cattle-, chicken-, and dog-associated markers, to investigate fecal contamination sources and their associations with enteric pathogens in rural water sources in Vhembe District, South Africa. Sresung et al. (2023) detected the marker in 25% of samples from a tropical drinking-water source. Kongprajug et al. (2020) found that qPCR analysis method could change reported concentrations by up to 1.95 log10 copies per 100 mL, illustrating the importance of analytical standardization. Browning et al. (2023) demonstrated use of Pig-2-Bac in a digital-PCR MST panel in Ozark streams.

Evidence for use in karstic systems remains limited. Zhang et al. (2014) evaluated a panel of Bacteroidales assays in karst springs and wells, including swine-associated targets. Because the exact contribution of the Pig-2-Bac assay defined by Mieszkin et al. (2009) requires verification against the original methods, this study should not be interpreted as a direct validation of Pig-2-Bac without confirmation.

Carter et al. (2026) evaluated Pig-2-Bac by qPCR in well, surface, cenote, and tap-water samples from nine Mayan communities in Yucatán, Mexico. Pig-2-Bac was detected in 4 of 21 well-water samples and 1 of 7 surface-water samples; no detections were reported in six cenote samples or one tap-water sample. The study reported prolonged holding times and suboptimal storage conditions during shipment, which may have affected marker detection. These findings provide preliminary evidence of field application in regional water sources and should be interpreted cautiously.

Ohad et al. (2015) provided an additional karst-spring context for swine-associated Bacteroidales markers. Xu et al. (2020) reported 100% sensitivity and specificity for the TaqMan Pig-1-Bac and Pig-2-Bac assays in a Chinese host reference collection, whereas SYBR Green variants showed cross-amplification with non-swine sources. Rytkönen et al. (2021) reported greater than 95% specificity for the DNA-based assay under its study conditions, whereas the rRNA-based approach increased sensitivity at the cost of reduced specificity. Monteiro et al. (2023) reported high concentrations of swine-associated Bacteroidales markers in a multicenter European study, although its concentration units differed from most other reports and are not directly comparable.

3.3.3 Pig-Bac-2

Heaney et al. (2015) evaluated Pig-Bac-2 alongside Pig-1-Bac, Pig-2-Bac, and P23-2 in a study of surface waters proximal to swine concentrated animal feeding operations in North Carolina, USA. Using conventional PCR, Pig-Bac-2 showed 93% sensitivity in the mixed collection of 15 swine-source samples and 37% specificity in 19 non-swine fecal samples. Its low specificity limited its ability to distinguish swine from other animal sources of fecal contamination. No other included study provided extractable numerical Pig-Bac-2 diagnostic estimates; the PF163 estimates reported by Lamendella et al. (2009) were not treated as Pig-Bac-2 evidence because equivalence between the two assays was not established.

3.3.4 P23-2

P23-2 targets the archaeal *mcrA* gene associated with swine fecal material and was originally described by Ufnar et al. (2007) using conventional PCR. In that study, the assay detected 84% of swine fecal samples and 100% of swine-waste-lagoon water samples; one of 260 non-swine samples amplified, corresponding to a specificity of 99.6%.

In an independent evaluation, Lamendella et al. (2009) reported diagnostic sensitivity of 49% and diagnostic specificity of 93% for P23-2. The article separately described 53% fecal positivity, which should not be substituted for the diagnostic sensitivity estimate. Heaney et al. (2015) did not detect P23-

2 by conventional PCR in any known-source sample (0/15 swine-source and 0/19 non-swine samples) or surface-water sample; the 53% detection frequency reported in that study referred to Pig-2-Bac during winter months and is an environmental detection frequency, not a P23-2 estimate or a measure of diagnostic sensitivity.

These findings indicate variable P23-2 performance across studies and outcome definitions. Its archaeal target provides a biologically distinct line of evidence from bacterial Bacteroidales markers, making it a potential complementary assay for source attribution. However, the variable sensitivity reported in independent evaluations does not support its use as a standalone primary screening marker.

3.4 Comparative performance of markers

Supplementary Table 2 compares the four markers across biological target, assay format, reported diagnostic-performance data, field application, cross-amplification, persistence, and geographic context. Figure 2 descriptively displays 16 study–marker–reference-collection entries from 10 unique publications. Entries were selected by a uniform rule adopted during manuscript revision rather than prespecified in the protocol: all verified and extractable sensitivity and/or specificity estimates for the four candidate markers in the included studies, distinguished by analytical platform and reference collection. When a study reported both a full reference collection and a subset, only the full collection was plotted. Diagnostic estimates that were not extracted or could not be verified against the full text were not displayed. Pig-1-Bac showed high reported performance in the host reference collections evaluated but had a smaller field-application record. Evidence for Pig-Bac-2 was sparse, whereas P23-2 showed variable fecal-detection estimates but may provide complementary evidence because it targets an archaeal gene. Comparative interpretation was descriptive because study designs and outcome definitions were heterogeneous.

4. Discussion

4.1 Principal findings and marker selection

This systematic review synthesized evidence from 34 primary studies published between 2007 and 2026 evaluating the analytical performance and field application of four swine-associated molecular markers —Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2—for fecal source tracking in environmental-water matrices. Within the evidence identified, Pig-2-Bac had the broadest field-application record across the range of environmental-water matrices and geographic settings represented in the included studies, including surface water, groundwater, runoff, coastal water, recreational water, drinking-water sources, and aquatic microcosms.

The available evidence therefore supports Pig-2-Bac as the best-supported candidate for initial screening of swine-associated fecal contamination in environmental water. This conclusion is relative to the studies identified in this review and does not imply universal superiority across locations, host assemblages, sample-processing protocols, or analytical platforms. Local validation against

representative swine and non-swine fecal reference collections remains necessary before operational implementation, particularly in tropical and karstic systems where local validation evidence remains limited.

Pig-2-Bac was supported by studies spanning multiple environmental-water matrices and geographic settings. Its evidence base included field applications, analytical validations, and controlled aquatic-microcosm persistence studies. These findings support its prioritization for local validation and initial screening, while recognizing that detection frequencies, concentrations, and diagnostic metrics cannot be compared directly across studies.

Pig-1-Bac showed high reported performance in the limited host reference collections evaluated but had a smaller field-application record than Pig-2-Bac. Pig-Bac-2 had a sparse evidence base, with insufficient independent environmental-water data to support use as a standalone primary marker. P23-2 showed lower and more variable fecal-detection estimates across independent evaluations. Its biologically independent archaeal target may provide complementary evidence, but the currently available evidence does not support P23-2 as a standalone primary screening assay.

Performance estimates were not formally pooled because of substantial heterogeneity in host reference collections, assay formats, sample processing, environmental matrices, analytical thresholds, and outcome definitions. Thus, any comparative interpretation reflects the breadth and consistency of the available evidence rather than a formal ranking of diagnostic accuracy.

4.2 Application in karstic water systems

Evidence for swine-associated MST assays in karstic water systems remains limited and heterogeneous. Zhang et al. (2014) evaluated a panel of seven Bacteroidales assays in karst springs and wells across four Midwestern United States. The study reported higher contamination in springs than in wells and detection of swine-associated and human-associated signals in co-contaminated karst groundwater systems. However, the specific contribution of the Pig-2-Bac assay defined by Mieszkin et al. (2009), relative to other swine-associated Bacteroidales assays, should be verified before the study is interpreted as direct Pig-2-Bac validation in karst. Ohad et al. (2015) provided an additional karst-spring context for swine-associated Bacteroidales markers in Israel. Together, these studies indicate that host-associated MST assays can be applied in karstic settings, but they do not establish transferability of Pig-2-Bac performance from temperate karst systems to tropical cenotes or groundwater networks. Carter et al. (2026) provided preliminary regional evidence of Pig-2-Bac application in well and surface-water samples from Mayan communities in Yucatán. However, the small sample size and prolonged holding times under suboptimal storage conditions limit interpretation of the observed detection pattern. These findings should therefore be viewed as an initial field application rather than as local assay validation for cenotes or karst groundwater. Accordingly, the available karst-related evidence supports local validation of Pig-2-Bac in tropical karst systems rather than its uncritical adoption as an already validated assay for cenotes and vulnerable groundwater systems in the Yucatán Peninsula.

4.3 The complementary value of Pig-1-Bac

Although Pig-2-Bac had a broader field-application record than Pig-1-Bac among the studies identified, Pig-1-Bac may provide useful confirmatory information when co-applied with Pig-2-Bac. The two assays target different sequences within the Bacteroidales 16S rRNA gene region; therefore, concordant detection may increase confidence in attribution of fecal contamination to swine. This approach is consistent with the general MST principle of using more than one host-associated line of evidence when source attribution requires high confidence (Harwood et al., 2014).

The available validation studies reported high Pig-1-Bac sensitivity and specificity in the host reference collections evaluated. However, its field-application evidence base was smaller than that identified for Pig-2-Bac, and its performance should not be assumed to be equivalent across geographic regions or host populations. Where laboratory resources permit, local validation of Pig-1-Bac alongside Pig-2-Bac may help determine whether a dual-marker strategy improves source-attribution confidence, diagnostic performance, or operational reliability in the intended monitoring setting.

4.4 The role of P23-2 as a potential triangulation marker

P23-2 has a phylogenetically distinct biological target because it amplifies the archaeal mcrA gene rather than a bacterial Bacteroidales 16S rRNA sequence. Consequently, co-detection of P23-2 with Pig-2-Bac may provide biologically independent supporting evidence for swine-associated fecal contamination, particularly in complex environmental matrices.

However, reported P23-2 fecal-detection estimates varied across the independent evaluations identified in this review, corresponding to distinct outcome definitions: Ufnar et al. (2007) reported detection in 84% of swine fecal samples, whereas Lamendella et al. (2009) separately reported 53% fecal positivity and a diagnostic sensitivity of 49%. These estimates were not combined into a single range. Evidence of its use in recent environmental-water field studies was also limited. P23-2 should therefore be considered a potential supplementary assay rather than a standalone primary screening marker. Its incremental value for source attribution, particularly in combination with Pig-2-Bac and Pig-1-Bac, should be evaluated prospectively using locally relevant swine and non-swine reference collections.

4.5 Methodological considerations

Differences in assay implementation can influence the interpretation and comparability of Pig-2-Bac results. Rytkönen et al. (2021) reported that rRNA-based detection increased sensitivity but reduced specificity for mammal-associated Bacteroidales markers under the conditions tested, underscoring the trade-off between assay sensitivity and specificity. Kongprajug et al. (2019) reported agreement of 87.5–100% between conventional PCR and qPCR results in one tropical river setting, whereas Kongprajug et al. (2020) showed that qPCR data-analysis approach could change estimated concentrations by up to 1.95 log10 copies per 100 mL. These results emphasize the need to standardize sample processing, amplification controls, thresholds, and quantitative-analysis procedures before comparing results across laboratories or monitoring programs.

The original TaqMan qPCR assay for Pig-2-Bac described by Mieszkin et al. (2009) has the broadest field-application record among the assay formats identified in this review. However, this review did not conduct a head-to-head, pooled comparison of assay formats; therefore, no assay platform can be regarded as universally optimal. Local analytical validation should assess sensitivity, specificity, inhibition, detection limits, extraction recovery, and cross-amplification against locally relevant host-source collections.

4.6 Limitations of the evidence base and review process

This review has several limitations. First, included studies were heterogeneous in host reference collections, assay formats, extraction procedures, environmental matrices, sample volumes, analytical thresholds, and definitions of diagnostic performance. This heterogeneity precluded formal meta-analysis and direct numerical ranking of assays. Second, title and abstract screening was initially conducted by one reviewer. To strengthen the reliability of the final inclusion decisions and extracted data, a second reviewer independently reassessed the eligibility of all full-text reports and verified the completeness and accuracy of data extraction and the descriptive appraisal of reporting completeness and methodological transparency. Third, the protocol was registered retrospectively after data extraction had begun. The search strategy was subsequently expanded to include Web of Science and forward and backward citation chasing; these amendments were documented in the OSF record and supplementary materials. Fourth, Scopus was not searched because institutional access was unavailable. Relevant studies indexed only in Scopus may not have been retrieved. Fifth, no formal risk-of-bias instrument was applied because the included studies encompassed heterogeneous analytical-validation, field-application, persistence, and methodological-comparison designs. Reporting completeness and methodological transparency were instead appraised descriptively (Supplementary Table 3). Differences in sample size, host reference-collection diversity, geographic scope, analytical controls, and assay-reporting completeness may have influenced reported performance estimates.

Finally, tropical and karstic groundwater systems remain underrepresented. Carter et al. (2026), a pilot study in Yucatán, had a small sample size and suboptimal sample-storage conditions, and the available temperate karst studies may not be hydrologically comparable to tropical cenote systems. Further local validation is therefore required before any marker is adopted for routine surveillance in those environments.

4.7 Implications for environmental-water monitoring

For environmental pollution management, MST results should be interpreted as evidence supporting source attribution rather than as standalone measures of microbial risk. Integration with conventional fecal indicators, hydrological information, land-use data, and pathogen monitoring, where feasible, can improve identification of contamination pathways and prioritization of mitigation measures in watersheds affected by swine production. Long-term monitoring in paired agricultural watersheds in Ontario, Canada, illustrates the use of MST to evaluate management interventions: Wilkes et al. (2014)

reported lower occurrence of a combined livestock-associated Bacteroidales category (ruminant and pig markers) under controlled than conventional tile drainage.

Within the evidence identified, Pig-2-Bac is the best-supported candidate for initial screening of swine-associated fecal contamination in environmental water. Where local validation and resources permit, co-application of Pig-1-Bac may strengthen confidence in source attribution. P23-2 may provide phylogenetically independent supporting evidence in selected applications but should not be used as a standalone primary assay. Controlled persistence findings should not be translated directly into field persistence or time since contamination, particularly in karst aquifers and cenote systems.

Future work should prioritize local validation against representative swine and non-swine fecal source collections; standardized extraction, inhibition-control, and qPCR/dPCR quantification procedures; assessment of marker fate and transport under tropical karst hydrology; development of in-region analytical capacity; and prospective comparison of single-marker and dual-marker monitoring strategies.

5. Conclusions

This systematic review synthesized evidence from 34 primary studies evaluating Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2 for swine-associated fecal source tracking in environmental water. Pig-2-Bac had the broadest field-application record across freshwater, groundwater, runoff, coastal, recreational, and drinking-water-source matrices, and its evidence base included controlled aquatic-microcosm persistence studies.

Within the evidence identified, Pig-2-Bac is the best-supported candidate for initial environmental monitoring of swine-associated fecal contamination. Pig-1-Bac may be a useful complementary co-marker where local validation and analytical capacity permit. P23-2 may provide phylogenetically independent supporting evidence but is not supported as a standalone primary assay, whereas the available evidence for Pig-Bac-2 is insufficient for standalone application.

Implementation should include local validation against representative swine and non-swine reference collections and standardized extraction, inhibition-control, and qPCR/dPCR procedures. Evidence remains limited for tropical karst groundwater and cenote systems; therefore, local validation and fate-and-transport studies should precede routine implementation in these vulnerable environments.

Declarations

Competing interests

The authors declare that they have no financial or non-financial competing interests.

Ethics approval

Not applicable. This study synthesized data from published literature and did not involve human participants, animals, or collection of new biological samples.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Clinical trial number

Not applicable.

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Author contributions

Mariel Gullian Klanian: Conceptualization, Methodology, Investigation, Search strategy, Study selection, Data curation, Formal analysis, Visualization, Project administration, Writing – original draft, and Writing – review & editing. María José Sánchez Solís: Methodology, Validation, Independent verification of full-text eligibility, Verification of data extraction, Verification of the reporting-completeness and methodological-transparency appraisal, Interpretation of results, and Writing – review & editing. Both authors read and approved the final manuscript.

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Data availability

The study-level findings synthesized in this systematic review are presented in the article and Supplementary Tables 1–3. The original data are available in the cited primary publications.

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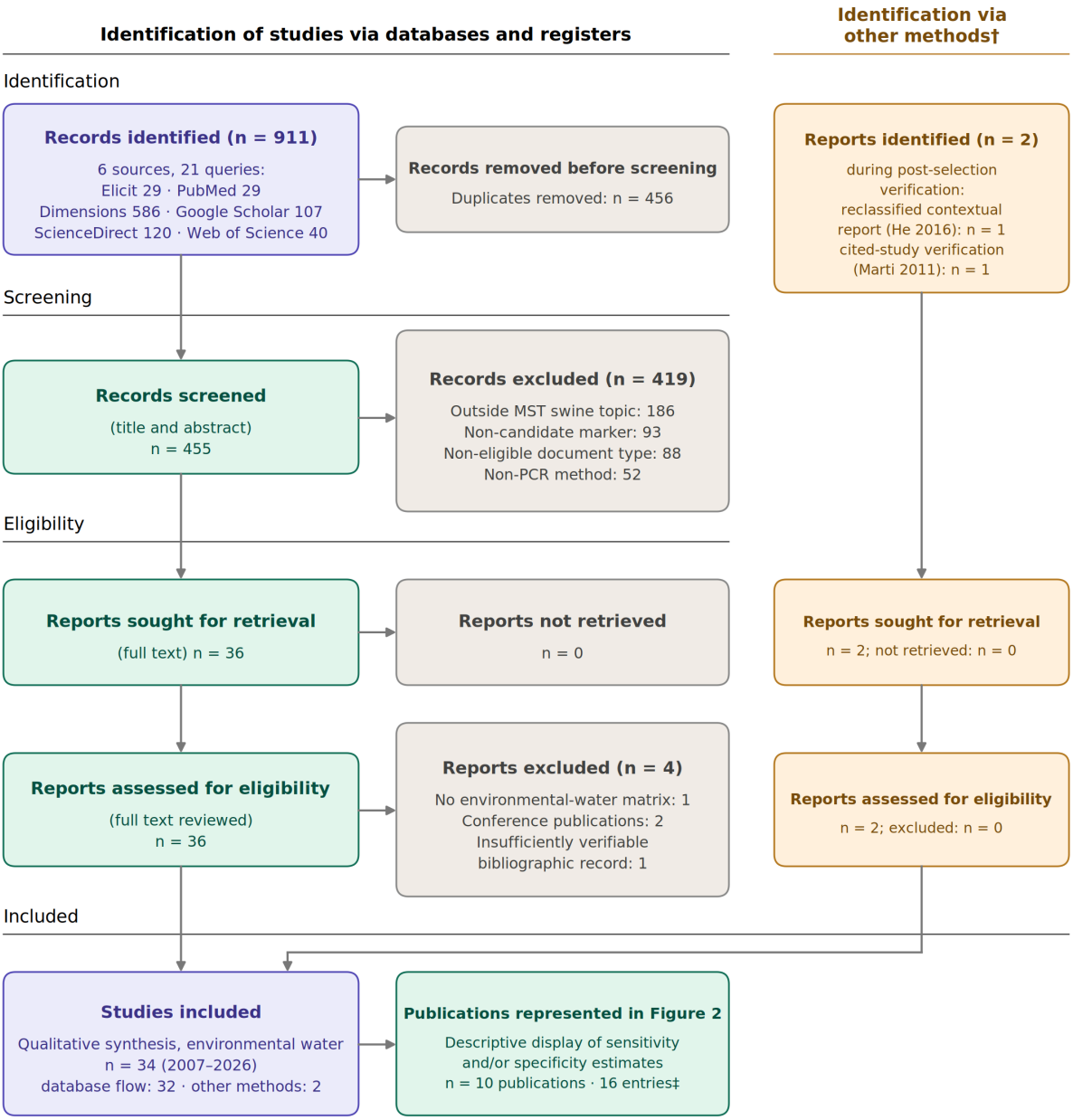
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Figures



PRISMA 2020 — Swine-associated molecular markers for fecal source tracking in environmental water

All searches: September 30, 2026 · OSF registration: osf.io/rx3y5 (retrospective) · Scopus not searched (access unavailable)

›nstruction (October 19, 2026) prepared for the revision package; the PRISMA flow diagram was not included with the original submission (ESPR-S-26-10835

tion based on current review documents; original screening records for He et al. (2016) and Marti et al. (2011) are not available. Database-flow counts are ι

Four contextual reports were not counted: Schiaffino et al. (2020), Pisanic et al. (2015), Kurowski et al. (2025), and Liu et al. (2023).

‡ AUC, PPV, NPV, environmental detection frequencies, concentrations, and prevalence ratios were not treated as sensitivity or specificity. No pooled estimates were calculated.

Figure 1

PRISMA 2020 flow diagram of study identification, screening, eligibility assessment, and inclusion. Searches across six sources using 21 queries identified 911 records. After removal of 456 duplicates, 455 records were screened and 36 full-text reports were assessed for eligibility. Four reports were

excluded, yielding 32 initially included studies. Post-selection verification added Marti et al. (2011) and reclassified He et al. (2016), bringing the final qualitative synthesis to 34 studies. The post-selection pathway was reconstructed retrospectively from current review documents because the original screening records for these two reports were unavailable; the database-flow counts remain unchanged.

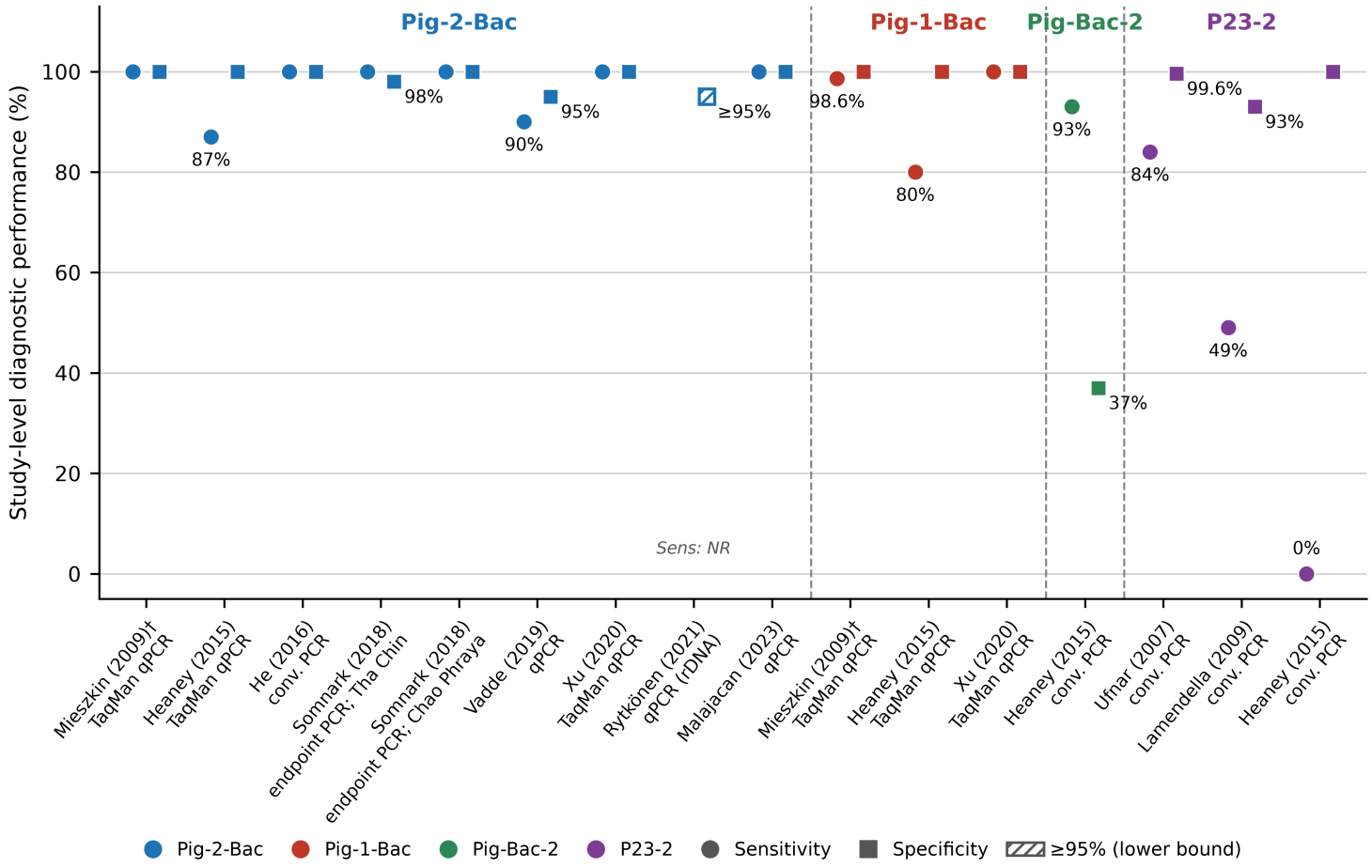


Figure 2

Study-level sensitivity and specificity estimates for swine-associated microbial source-tracking markers in environmental-water research. Circles indicate sensitivity and squares indicate specificity; blue denotes Pig-2-Bac, red denotes Pig-1-Bac, green denotes Pig-Bac-2, and purple denotes P23-2. The display includes 16 study–marker–reference-collection entries from 10 publications, selected by a uniform rule adopted during manuscript revision: all verified and extractable sensitivity and/or specificity estimates for the four candidate markers in the included studies. Each entry is labeled by analytical platform. Reference collections: Mieszkin et al. (2009), 69 mixed swine-source samples (Pig-2-Bac 69/69; Pig-1-Bac 68/69, approximately 98.6%, calculated from the original Table 2) and 54 non-swine fecal samples (54/54 negative), with 100% sensitivity for both markers in the 25 individual swine fecal samples; Heaney et al. (2015), 15 mixed swine-source samples (feces, wallow water, waste-lagoon water) and 19 non-swine fecal samples, with Pig-1-Bac and Pig-2-Bac tested by TaqMan qPCR and Pig-Bac-2 and P23-2 by conventional PCR; P23-2 was not detected in any known-source sample, so 0% sensitivity (0/15) and 100% specificity (19/19) were calculated from the reported collection sizes; He et

al. (2016), 25 swine and 107 non-swine fecal samples tested by conventional PCR; Somnark et al. (2018), two watershed-specific host collections; Vadde et al. (2019), Xu et al. (2020), Malajacan et al. (2023), Ufnar et al. (2007), and Lamendella et al. (2009), as described in Supplementary Table 1. Rytkönen et al. (2021) is displayed at 95% to represent the reported specificity lower bound (≥95%), not an exact estimate. Lamendella et al. (2009) reported 49% diagnostic sensitivity and 93% diagnostic specificity for P23-2. Subset estimates (for example, Heaney et al. after exclusion of wallow-water samples) were not plotted separately. The PF163 estimate reported by Lamendella et al. (2009) was not plotted because its equivalence with Pig-Bac-2 was not established. Within each marker, entries are ordered chronologically. PPV, NPV, AUC/accuracy, environmental detection frequencies, concentrations, method agreement, prevalence ratios, and persistence estimates were not plotted as sensitivity or specificity. No pooled estimates, confidence intervals, or summary statistics were calculated because reference collections, assay formats, matrices, and outcome definitions were heterogeneous. Symbols are slightly offset horizontally within each entry to avoid overlap.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable2.docx
- SupplementaryTable3.docx