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Pig-2-Bac as a biomarker of occupational exposure to pigs and livestock-associated *Staphylococcus aureus* among industrial hog operation workers

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Abstract

Over 50 million hogs are raised annually in the United States for consumption, mostly on industrial hog operations (IHOs). Workers at IHOs are exposed to airborne particulates, zoonotic pathogens, and other workplace hazards, but lack of access to IHOs can hinder exposure assessment in epidemiologic studies. Here, we demonstrate the utility of pig-specific *Bacteroidales* (Pig-2-Bac) as a biomarker of exposure to pigs and pig waste and to help identify sources of *Staphylococcus aureus* carriage among IHO workers.

Keywords

Pig-specific fecal *Bacteroidales*; Quantitative polymerase chain reaction; Livestock-associated *Staphylococcus aureus*; Occupational exposure; Industrial hog operation

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Competing interests

SW authored a report in a civil rights complaint against the NC Department of Environment and Natural Resources' 2014 re-permitting of industrial hog operations in NC, and a report on behalf of plaintiffs in a civil case regarding impacts of IHOs on neighbors. He has not received or requested compensation in either matter. All other authors declare no competing interests.

1. Introduction

Over 50 million hogs are raised annually in the United States for consumption, mostly on industrial hog operations (IHOs) (USDA, 2015). Workers at IHOs may be exposed to a variety of hazards in the workplace, including airborne particulates and zoonotic pathogens such as certain strains of antibiotic-resistant and livestock-associated *Staphylococcus aureus* (Nadimpalli et al., 2015b; Rinsky et al., 2013) and swine-origin influenza viruses (Gray et al., 2007). In Europe and other regions of the world, members of the public health research community have been able to sample livestock production workplace environments, animal herds and workers on-the-job to investigate conditions that can expose workers to zoonotic pathogens. However, limited access to IHOs in the US impedes direct exposure assessment and epidemiologic investigations of the sources of zoonotic pathogen exposure among US IHO workers. Instead, IHO workers' self-reports of exposure are often used as indicators in epidemiological studies, however, workers' perception of the frequency, intensity, and duration of microbial exposure may vary between workers and within workers over time, leading to exposure misclassification. An alternative method to identify occupational microbial exposure burdens is to develop exposure biomarkers using existing microbial source tracking methods. Microbial source tracking has been used to identify the sources of human and animal fecal pollution in the environment, primarily in water (Boehm et al., 2013). One biomarker that has been used to track swine fecal microbial contamination in surface water is Pig-2-Bac, a pig-specific *Bacteroidales* marker (Heaney et al., 2015; Mieszkin et al., 2009). Such biomarkers could provide a more objective estimate of microbial exposure than IHO workers' self-reports and therefore could improve exposure assessment even in settings with complete access to the workplace.

We estimated associations between Pig-2-Bac isolated from nasal swabs collected among IHO workers and time since last work shift to determine whether Pig-2-Bac could be a useful biomarker of occupational exposure to pigs and pig waste. We then assessed associations between Pig-2-Bac and workers' nasal carriage of a *S. aureus* gene (*femA*), a methicillin resistance gene (*mecA*), and culture-based livestock-associated vs. human-associated *S. aureus* nasal carriage status (persistent, intermittent, non-carrier).

2. Methods

Twenty-two IHO workers were enrolled between June–August, 2012 in a 14-day repeated measures study described previously (Nadimpalli et al., 2015b). Workers self-collected a dual-tipped nasal swab (CultureSwab™ Liquid Stuart, BD Diagnostics) on the evening of day 1, and each morning and evening on days 2–7 and 14. Up to 15 swabs were collected per IHO worker. Each time participants collected a nasal swab, they recorded collection time (morning or evening) and whether they worked that day or not. One swab of each pair was tested for culturable *S. aureus* using previously described methods (Nadimpalli et al., 2015b) and *S. aureus* isolates from nasal swabs were assessed for antibiotic susceptibility, *spa* type and absence of the *scn* gene to assess livestock vs. human association. Livestock association was defined by absence of *scn* (Price et al., 2012; Stegger et al., 2013).

2.1. Molecular analysis

DNA was extracted from nasal swabs using a Qiagen Viral RNA Mini kit (Valencia, CA), which co-purifies RNA and DNA. The spin protocol was used according to the manufacturer's instruction with the exception that clipped nasal swabs were immersed in the recommended volume of lysis buffer during the lysis step (Nadimpalli et al., 2015a). Nucleic acid extracts were assessed by real-time qPCR for the *S. aureus* specific gene *fem*A, the methicillin resistance gene *mecA* (Francois et al., 2003), and Pig-2-Bac (Mieszkin et al., 2009). Two μL of each nucleic acid extract were used as template in a 20 μL qPCR reaction containing 10 μL 2X reaction mix (TaqMan® Fast Advanced Master Mix, Applied Biosystems, Foster City, CA), 500 nM forward and reverse primers and 250 nM dual-labeled probe. All samples were analyzed on a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) using 40 amplification cycles. Each cycle consisted of a denaturation step (20 s at 95 °C), an annealing step (20 s at 56 °C for *mecA* and 60 °C for *femA* and Pig-2-Bac) and an amplification step (20 s at 60 °C). A quantitative standard curve using *femA* or *mecA* containing plasmid standards with known gene copy numbers was included in each *femA* and *mecA* qPCR. In each Pig-2-Bac qPCR we included *Bacteroidales* DNA isolated from a NC pig lagoon sample in duplicate as a positive control (C_T = 31.0 ± 0.1). Additionally, non-template negative controls (nuclease-free water) were included in each qPCR. *femA* and *mecA* were quantified using the StepOne™ software version 2.3. For Pig-2-Bac, relative qPCR estimates were calculated based on the observed C_T (cycle threshold) using the formula in Eq. (1).

$$\Delta C_T = 2^{(40 - \text{swab } C_T)} \quad (1)$$

2.2. Statistical analysis

Samples below the limit of detection for *femA* and *mecA* were assigned a value of ½ the lowest detectable qPCR estimate and for Pig-2-Bac were assigned a value of $\Delta C_T = 1$ (i.e. $C_T = 40$). All qPCR estimates were $\log_{10}$ transformed. Unity-based normalization of the $\log_{10}$ transformed qPCR estimates was used to scale each participant's qPCR estimates to a range between 0 and 1 using the formula in Eq. (2) (Aksoy and Haralick, 2001).

$$x' = \frac{x - \min(x)}{\max(x) - \min(x)} \quad (2)$$

This normalization reduces the potential influences introduced by workers at the extremes of the qPCR target distribution – e.g., persistent *S. aureus* carriers (with high *femA* qPCR estimates) compared to non-carriers (with low *femA* qPCR estimates). We graphed associations between time since last IHO shift and Pig-2-Bac qPCR estimates by plotting the mean ± standard error (SE) of the $\log_{10}$ and unity-normalized qPCR estimates by days since last IHO shift. We also graphed associations of Pig-2-Bac with *femA* and *mecA* qPCR estimates by plotting the $\log_{10}$ mean ± SE and unity-normalized mean ± SE *femA* and *mecA* qPCR estimates by tertile of the Pig-2-Bac qPCR estimates. Graphs were produced with Matlab R2015a (The MathWorks, Inc., Natick, MA).

We used fixed effect linear regression models, which account for non-independence of repeated measurements within person (Allison, 2005), to estimate beta coefficients and SEs of associations between time since last IHO shift and Pig-2-Bac qPCR estimates and between Pig-2-Bac and *femA* and *mecA* qPCR estimates. Finally, we used multinomial logistic regression models to estimate odds ratios (OR) and 95% confidence intervals (CI) of the associations of (1) mean Pig-2-Bac qPCR estimates and (2) mean time since last work shift with livestock-vs. human-associated *S. aureus* carriage status (persistent and intermittent compared to non-carriage of culture-based *S. aureus*). For each type of *S. aureus* (i.e., livestock-associated *S. aureus*; human-associated *S. aureus*), respectively, IHO workers who carried at each or each minus one sampling point were classified as persistent carriers, workers who never carried were classified as non-carriers, and remaining workers were classified as intermittent carriers. The non-carrier category was used as the referent group in multinomial logistic regression analyses. Regression analyses were completed using SAS version 9.3 (SAS Institute, Cary, NC).

3. Results and discussion

Twenty-two North Carolina IHO workers provided 327 nasal swabs; 316 were dual swabs with one swab available for qPCR analysis. Swabs were collected each morning and evening, regardless of whether the participant worked that day. Most samples (76%) were collected <1 day after an IHO work shift (*n*= 221). Of those, 55% were collected in the evening after an IHO work shift and 45% were collected the following morning. Thirteen percent of samples (*n*= 39) were collected ≥1 and <2 days after an IHO work shift (after one day off), 9% (*n*= 26) were collected ≥2 and <3 days after an IHO shift (after two days off), and 2% of samples (*n*= 6) were collected ≥3 days after an IHO shift (after 3 or more days off).

3.1. Association of Pig-2-Bac with time since last work shift

Pig-2-Bac was detected in 61% of nasal swab samples (mean ± SE Pig-2-Bac $\log_{10}$ ΔC_T/swab = 1.5 ± 0.8). Pig-2-Bac mean $\log_{10}$ and normalized qPCR estimates declined with increasing time since IHO shift (Fig. 1). Fixed-effects linear regression modeling also showed that $\log_{10}$ Pig-2-Bac decreased 0.35 (SE: 0.07) ΔC_T/swab per day since last work shift (Table 1a). This negative association suggests that Pig-2-Bac is a useful biomarker for human exposure to pigs or pig waste during IHO work.

3.2. Association of Pig-2-Bac with S. aureus and methicillin resistance

We next estimated associations of Pig-2-Bac with *femA* and *mecA* qPCR estimates. The *femA* gene was present in 67% of nasal swab samples (mean ± SE $\log_{10}$ copy number/swab = 4.2 ± 1.3) and *mecA* was present in 93% of nasal swab samples (mean ± SE $\log_{10}$ copy number/swab= 4.7 ± 0.9). Both mean $\log_{10}$ and unity-normalized *femA* and *mecA* qPCR estimates increased by Pig-2-Bac tertile, although *mecA* qPCR differences were less pronounced between the 2nd and 3rd tertile than *femA* differences (Fig. 2). Fixed-effects linear regression modeling also showed that *femA* and *mecA* increased by 0.14 (SE:0.06) and 0.09 (SE:0.04), respectively, for every one-unit change in Pig-2-Bac (Table 1b–c). These associations suggest that intensive contact with pigs or pig waste (reflected by high Pig-2-

Bac estimates in the nares) may be the source of IHO worker exposure to *S. aureus* (*femA*) and methicillin resistance (*mecA* is carried by diverse bacteria).

3.3. Association of Pig-2-Bac with livestock-versus human-associated S. aureus nasal carriage status

Finally, we examined whether mean Pig-2-Bac qPCR estimates (as a proxy for intensity of contact with pigs or pig waste during IHO work) and mean time since last work shift (as a proxy for time off from work) was associated with nasal carriage status of livestock- or human-associated *S. aureus*. In this study, 10 workers carried livestock-associated *S. aureus* strains persistently, and two carried human-associated *S. aureus* strains persistently. Analyses of associations of mean Pig-2-Bac qPCR estimates with livestock-associated *S. aureus* nasal carriage status (persistent, intermittent, non-carrier) showed higher odds ratios for intermittent (OR: 1.8; 95% CI: 0.2, 19.8) and persistent (OR: 12.8; 95% CI: 1.0, 160.6) carriage compared to non-carriage (Table 2a). We did not observe associations between mean Pig-2-Bac and human-associated *S. aureus* carriage status (Table 2a). We also did not see associations between mean time since last work shift and either livestock- or human-associated *S. aureus* carriage status (Table 2b). These findings indicate that nasal carriage of livestock-associated *S. aureus* may be related to intensity of contact with pigs or pig waste during IHO work (as reflected by associations in Table 2a) and that not all IHO work may contribute equally as a source of exposure to livestock-associated *S. aureus* (as reflected by no association with increasing mean time since last work shift; Table 2b). Although the ORs presented in Table 2 are based on 22 people and therefore are imprecise, the directions of the associations found are informative.

Limitations of this study include the lack of a referent group (non-IHO worker group without exposure to pigs) to evaluate the specificity of Pig-2-Bac as a biomarker of exposure to pigs and pigs waste in human biospecimens. However, the specificity of Pig-2-Bac as a source tracking marker for pig fecal contamination in water has been validated previously and found to be 100% specific (Mieszkin et al., 2009). Other limitations included the lack of the exact nasal swab collection time; exact time the last IHO work shift ended and information about specific IHO work activities performed each day. The time since last work shift variable was therefore binned into 4 categories: <1 day since last work shift, 1–<2 days, 2–<3 days and 3+ days after last work shift. Even though the categories of the time since last shift variable are somewhat broad, Pig-2-Bac estimates declined significantly with days since last work shift.

Although self-reported time since last work shift or job tasks may be useful indicators for recent IHO work exposure, time since last shift alone often may not accurately reflect the intensity of exposure to pigs or to pig waste during specific job duties. Quantitative Pig-2-Bac estimates derived from nasal swab samples provide a more objective measure of exposure to high concentrations of pig microbes on-the-job than self-reported time since last work exposure. Additionally, participants' perception of the frequency, intensity, and duration of exposure to pigs or pig wastes on a specific day or during a specific job task may vary significantly and may lead to misclassification in epidemiological studies.

In summary, we found that pig-specific fecal *Bacteroidales* (Pig-2-Bac) is a useful biomarker of recent human occupational exposure to pigs or pig waste during IHO work. We showed that the application of Pig-2-Bac as a biomarker can provide evidence that the source of nasal carriage of *S. aureus* (*femA* gene) and methicillin resistance (*mecA* gene) may be intensive contact with pigs or pig waste at IHOs. We also showed that higher mean Pig-2-Bac qPCR estimates were associated with higher odds of persistent carriage of livestock-associated *S. aureus*. To our knowledge, this is the first study to apply Pig-2-Bac as a microbial source tracking marker for recent human exposure to pigs.

Although this study was small and the generalizability of its findings is unclear, its major strength is the repeated measures, which facilitate assessment of the temporality of a quantitative measure of a swine-specific microbial source tracking marker with both quantitative molecular and culture-based measures of *S. aureus* and antimicrobial resistance. The temporal dynamics we observed suggest that intensive contact with pigs can be a source of IHO worker *S. aureus* exposure relative to other potential *S. aureus* exposure sources at home or in the community.

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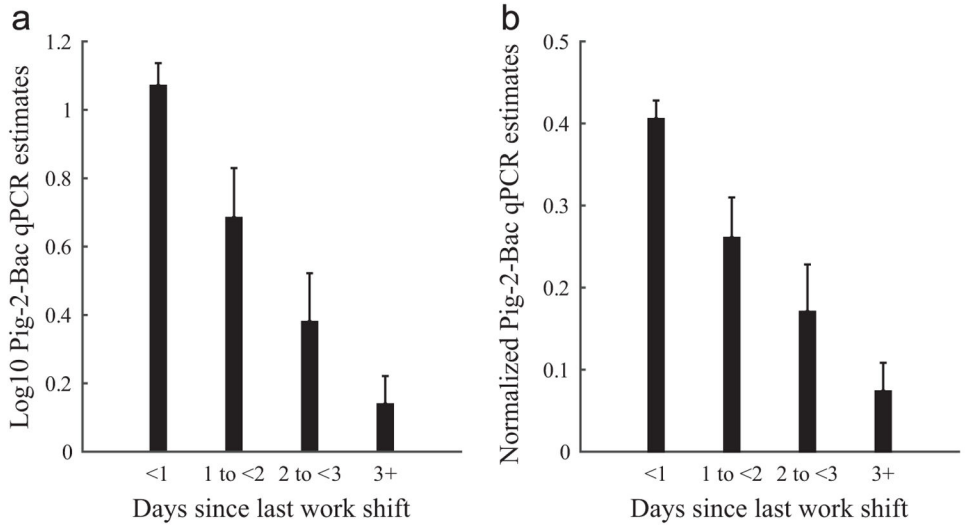

Fig. 1.

Relation of time since last work shift with (a) $\log_{10}$ transformed and (b) normalized by participant qPCR estimates of Pig-2-Bac DNA ΔC_T per nasal swab collected from 22 industrial hog operation workers in North Carolina, 2012. *Note.* qPCR= quantitative polymerase chain reaction. Error bars represent the standard error of the mean of the $\log_{10}$ transformed or normalized data, respectively.

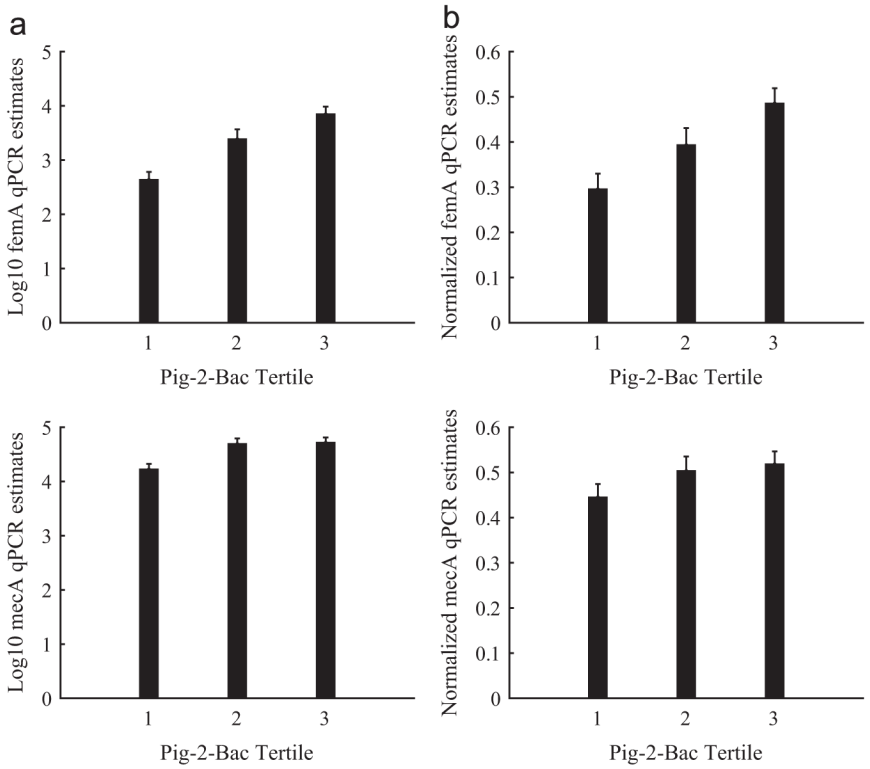

Fig. 2.

Relation of Pig-2-Bac qPCR estimates with $\log_{10}$ transformed (a) and normalized (b) qPCR estimates of *femA* and *mecA* gene copy number per nasal swab collected from 22 industrial hog operation workers in North Carolina, 2012. *Note.* qPCR= quantitative polymerase chain reaction. Error bars represent the standard error of the mean of the $\log_{10}$ transformed or normalized data, respectively.

Table 1

Relation of days since last work shift with Pig-2-Bac qPCR estimates and of Pig-2-Bac with *femA* and *mecA* qPCR estimates among 22 industrial hog operation workers in NC, 2012.

	N[a]	β (SE)	*t*	*p*
(a) Pig-2-Bac DNA ΔC_T per nasal swab				
Days since last work shift	289	−0.35 (0.07)[b]	−5.14	<0.01
(b) *femA* DNA copy # per nasal swab				
Pig-2-Bac DNA ΔC_T per nasal swab	313	0.14 (0.06)[c]	2.23	0.03
(c) *mecA* DNA copy # per nasal swab				
Pig-2-Bac DNA ΔC_T per nasal swab	313	0.09 (0.04)[c]	1.97	0.06

Beta coefficients are derived from fixed effects linear regression models. SE = standard error. C_T = cycle threshold. $\Delta C_T = 2^{(40 - \text{swab } CT)}$. qPCR = quantitative polymerase chain reaction.

[a] Ns represent the total number of observations included in the analysis for the days since last work shift (N missing = 24) and qPCR (N missing = 3) variables.

[b] The beta coefficient is the one $\log_{10}$ unit change in the Pig-2-Bac ΔC_T per nasal swab qPCR estimate for every one unit change in the days since last work shift variable (0–3 days).

[c] The beta coefficient is the one $\log_{10}$ unit change in the *femA* or *mecA* qPCR estimate for every one $\log_{10}$ unit change in the Pig-2-Bac ΔC_T per nasal swab qPCR estimate.

Table 2

Relation of mean Pig-2-Bac qPCR estimates and mean time since last work shift with *S. aureus* nasal carriage status among 22 industrial hog operation workers in North Carolina, 2012.

	a) Pig-2-Bac			b) Days since last IHO shift	
	N[d]	OR (95% CI)	*p*	OR (95% CI)	*p*
***S. aureus* nasal carriage status**[a]					
Livestock-associated[b]					
Non-carrier	6	ref		ref	
Intermittent carrier	6	1.8 (0.2, 19.8)	0.60	1.0 (0.0, 66.6)	0.99
Persistent carrier	10	12.8 (1.0, 160.6)	<0.05	2.0 (0.0, 80.0)	0.72
Human-associated[c]					
Non-carrier	16	ref		ref	
Intermittent carrier	4	0.2 (0.0, 2.1)	0.18	8.1 (0.1, 476.6)	0.31
Persistent carrier	2	0.7 (0.1, 10.2)	0.80	0.1 (0.0, 79.5)	0.55

Note. C_T = cycle threshold. $\Delta C_T = 2^{(40 - \text{swab } CT)}$. qPCR = quantitative polymerase chain reaction. OR = odds ratio. CI = confidence interval. Odds ratios were estimated using multinomial logistic regression models with non-carrier (0) as the referent group.

[a] *S. aureus* nasal carriage status is the dependent variable, coded as 0=non-carrier, 1=intermittent carrier, 2=persistent carrier.

[b] Defined as non-, intermittent or persistent nasal carriage of *scn* negative *S. aureus* strains

[c] Defined as non-, intermittent or persistent nasal carriage of *scn* positive *S. aureus* strains

[d] Number of non-, intermittent and persistent livestock- or human-associated *S. aureus* carriers in each category.