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Short Communications

Prevalence of Zoonotic Pathogens from Feral Pigs in Major Public Drinking Water Catchments in Western Australia

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Abstract: Australia has the largest number of wild pigs in the world. Their pronounced impacts on agriculture and biodiversity make the estimated 23 million feral pigs one of Australia's most important vertebrate pest species. The foraging and wallowing behavior of pigs can markedly increase the turbidity of water supplies, but more importantly, they can transmit and excrete a number of infectious waterborne organisms pathogenic to humans. Their persistence in drinking water catchments also makes them potentially significant reservoirs for zoonotic pathogens. In this study, important protozoan parasite pathogens, such as *Giardia*, *Cryptosporidium*, *Balantidium*, and *Entamoeba*, were detected from the feces of feral pigs caught in metropolitan drinking water catchment areas. All are potentially important waterborne human pathogens that pose a major threat to drinking water quality. Fortunately, the overall prevalence in feral pigs appears to be relatively low, with $\leq 13\%$ of pigs detected with parasites. In this study, we combined the findings from the parasitological analysis with the use of 14 highly informative DNA markers to define a series of highly structured populations that indicated very little movement of feral pigs between the populations. The implication of this pattern is that any public health risk may spread very slowly *between* populations, but may be much higher *within* watercourses. This study represents an innovative and important new approach to drinking water source protection in Australia.

Key words: *Balantidium*, *Giardia*, *Cryptosporidium*, waterborne pathogens, public health, *Sus scrofa*

Feral pigs (*Sus scrofa*) are seen as pests in many countries and have been labeled ''triple threat pests'' due to their impacts on agriculture, biodiversity, and human health (Choquenot et al., 1996; Hampton et al., 2004b). Pigs have a greater ability than most animal species to harbor and excrete important waterborne zoonotic infections (e.g., MacKenzie et al., 1994; Atwill et al., 1997), and combined with their destructive activities around permanent water sources, are probably an important reservoir and a target for intense management for the protection of source (drinking) water supplies (Atwill et al., 1997). While much has been made of the potential role of feral pigs as disease *reservoirs*, the waterborne pathogens they potentially excrete in water catchment areas have remained poorly understood. Despite considerable economic costs in feral pig control, large numbers of feral pigs generally remain either in these sensitive and intensively controlled catchment areas or reinvade relatively quickly from adjacent populations (see also Hampton et al., 2004a, b).

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The integrity and importance of a riparian buffer is well recognized in water management (Naiman and Decamps, 1997; Malard et al., 2002). The rooting behavior of feral pigs in moist soil is thought to markedly increase the turbidity of water bodies when pigs forage on the banks of creeks feeding into dams, which ultimately impacts on the deterioration/destruction of these riparian zones. Turbidity is a critical parameter of drinking water quality, and high levels can protect waterborne pathogens from the chemical disinfection used in water treatment (Agriculture and Resource Management Council of Australia and New Zealand, 2001). Pigs are the only source of *Balantidium coli* infection in humans who acquire the infection by drinking water containing environmentally resistant cysts (Karanis et al., 1993; Garcia, 1999; Schuster and Visvesvara, 2004). Similarly, *Giardia* and *Cryptosporidium* are of great public health significance and are well-documented waterborne agents of disease (Caccio et al., 2005). *Cryptosporidium* was responsible for infecting 400,000 people, and killing 12, in the United States following contamination of drinking water supplies (MacKenzie et al., 1994), and *Giardia* and *Cryptosporidium* were the cause of the 1996 Sydney water scare (Agriculture and Resource Management Council of Australia and New Zealand, 2001). In the broader scheme, feral pigs are considered to be one of the most important threats to livestock industries through their potential ability to act as reservoirs for a number of animal diseases exotic to Australia, including foot-and-mouth disease and surra (Choquenot et al., 1996; Thompson et al., 2003).

Here we report on the prevalence of zoonotic waterborne pathogens and the potential role of population structure in pathogen persistence and distribution among feral pigs. It is unique in linking both the identity of zoonotic agents and the population structure of the reservoir. These data were then used to facilitate an understanding of the movement patterns of these pests. Population genetics was considered a complementary and logical linkage to allow the elucidation of population structure, the identification of areas acting as reinvasion sources, and the identification of areas having suffered recent drops in population size. Additionally, modern genetic approaches are able to identify immigrant individuals (Hampton et al., 2004b), or those that may have been illegally released by humans (Spencer and Hampton, 2005). The parasite presence and genetics were used to identify potential zoonotic agents and to link these to the likely pattern and movement (gene flow) and consequences of endemic diseases in water catchments.

Fecal samples from 292 feral pigs, collected between 2003 and 2004 from metropolitan water catchment areas (Fig. 1), were analyzed for coproparasitoscopic analysis. Each feral pig fecal sample was subjected to zinc sulfate flotation and microscopy; additionally, staining with malachite green was used for the detection of *Cryptosporidium* (Bartlett et al., 1978; Elliot et al., 1999). Molecular characterization of *Giardia* and *Cryptosporidium* was performed as previously described (Becher et al., 2004).

Individual tissue samples for genetic analysis were collected from feral pigs at 11 sampling locations in the southwest corner of Australia between May 2001 and July 2003 (Fig. 1). In most instances, samples were opportunistic and collected during routine control operations by local pest animal management agencies and private landowners. Samples for this component of the study comprised a subgroup of the same individuals sampled for parasitological identification described above. Sampling involved the collection of a small tissue sample (skin, muscle, or liver in a solution of 20% dimethyl sulfoxide and sodium chloride) for molecular analysis. Basic demographic (weight, age, sex, date of collection) and location (global positioning system) information were collated for each animal. Complete DNA profiles were generated for all available (n = 147) adult feral pigs. Fourteen polymorphic microsatellite loci that had been shown previously to be highly informative for *S. scrofa* (Alexander et al., 1996) were used. These microsatellites have been characterized extensively in pigs (Martinez et al., 2000; Vernesi et al., 2003) and are highly polymorphic, not linked, with no indication of null-alleles (Alexander et al., 1996). When markers occurred on the same chromosome, they were chosen with a minimal distance of 30 centimorgans (cM). The 14 microsatellite loci were amplified using multiplex polymerase chain reaction (PCR). Briefly, reactions were carried out in a 10 µl volume under the following conditions: 50 ng template DNA, 10 ρmol of each primer (labeled with HEX, FAM, and TET fluorescent dyes), 0.6 U of Taq polymerase, 0.2 mM of each dNTP, 1× reaction buffer, 0.1 mM bovine serum albumin, and 1.5 mM $MgCl_2$. PCR conditions included an initial denaturation for 5 minutes followed by 35 cycles at 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. This was followed by an extension step at 72°C for 10 minutes. Multiplex reactions were loaded together. DNA fragments were separated on a 5% polyacrylamide gel using an ABI 377 automatic sequencer. Size was determined by co-running a size standard (TAMRA-350, Applied Biosystems, Melbourne, Australia).

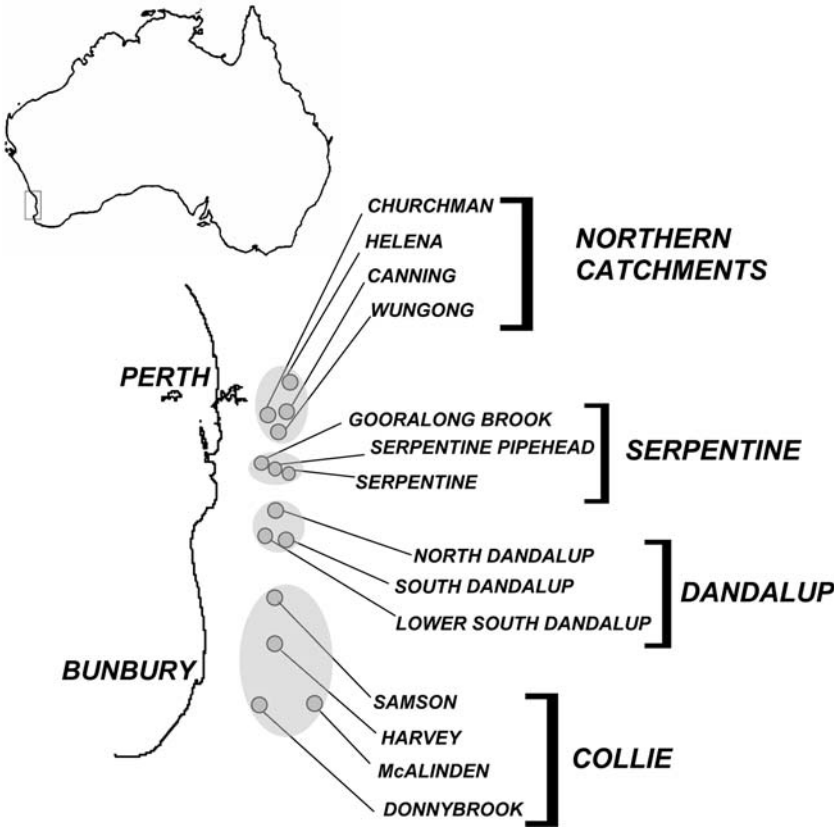

Figure 1. Relationship and distribution of feral pig populations from different regions in the Perth metropolitan and southwest drinking water catchment areas, generated using genetic markers.

DNA fragments were scored manually, with the aid of Genescan (Applied Biosystems, Melbourne, Australia).

A full Bayesian assignment approach according to Pritchard et al. (2000) was used to identify genetic structure, infer migration, and assign individuals to their likely population of origin using Structure. This approach was used to infer a *predefined* sample group is a discrete geographic area from which samples were obtained (Fig. 1), and an *inferred* population is a collection of sample groups that clustered together from the assignment results (Table 1). The results generated were based on simulations from 1 to 10 ($K = 1 - 10$) inferred populations, using a burn-in period of 50,000 iterations with 10^6 iterations of a Markov chain Monte Carlo simulation (see Pritchard et al., 2000).

Evidence of recent population bottlenecks was investigated by testing for an excess in heterozygosity, using the program Bottleneck (Piry et al., 1999). Due to the relatively small number of loci analyzed ($n = 14$), a Wilcoxon sign-rank test was estimated. A mixed model of microsatellite mutation was assumed, with single step mutations assumed to account for 90% of all mutation events, and a variance among multiple steps of 12, as suggested by Piry et al al. (1999) and Vernesi et al. (2003). The effective size for each population (N_e) was estimated from effective heterozygosity values, according to the methods for populations under mutation-drift equilibrium (Ohta and Kimura, 1973).

The hierarchical population structure was further defined by calculating F_{ST} (θ) values, using the program Fstat (Goudet, 2001). We used F_{ST} values to estimate the extent of gene flow and intrapopulation differences within and between water catchments. F_{ST} (θ), rather than R_{ST}, was used, as recommended by Gaggiotti et al. (1999), for studies involving small sample size and low numbers of loci being scored ($n < 20$). The effective number of migrants between any two inferred populations per generation (N_m) was estimated using F_{ST} values (Cockerham and Weir, 1993). All values are given as mean ± 1 SD.

Of 292 individuals sampled for parasite investigation, 10 specimens were identified with *Balantidium*, 9 with *Entamoeba*, 5 with *Giardia*, and 1 with *Cryptosporidium* (Table 2). Genotyping results from *Giardia* and *Cryptosporidium* isolated from feral pigs were inconclusive. Based on the results of Atwill et al. (1997), the strains detected by our study have zoonotic potential. Similarly, although *Chilomastix mesnili* and *Entamoeba coli* are known to infect humans, their clinical significance appears to be minimal

Table 1. Estimated Genetic Proportion of Each of the Four Inferred Population Clusters in Each of the 11 Sample Groups Generated Using Structure (Pritchard et al., 2000)[a]

			Inferred population/sample site			
	H_o (± SD)	H_e (± SD)	1	2	3	4
Perth Hills (n = 29)	0.586 ± 0.153	0.608 ± 0.096				
1. Helena			**0.618**	0.051	0.068	0.026
2. Canning			**0.605**	0.013	0.052	0.069
3. Churchman			**0.911**	0.013	0.031	0.008
4. Wungong			**0.952**	0.004	0.011	0.006
Serpentine (n = 46)	0.637 ± 0.101	0.618 ± 0.094				
5. Serpentine			0.150	**0.736**	0.032	0.026
Dandalup (n = 56)	0.638 ± 0.137	0.643 ± 0.098				
6. North Dandalup			0.012	0.004	**0.827**	0.015
7. South Dandalup			0.170	0.024	**0.539**	0.130
Collie (n = 16)	0.484 ± 0.207	0.539 ± 0.177				
8. Samson Brook			0.014	0.017	0.016	**0.833**
9. Harvey			0.031	0.023	0.049	**0.410**
10. Donnybrook			0.006	0.010	0.007	**0.921**
11. McAlinden			0.017	0.007	0.015	**0.682**

[a]Values in boldface indicate the most likely inferred population of origin (see also Fig. 1), and n indicates the number of individuals tested. Also included are basic measures of genetic variability: mean observed heterozygosity (H_o) and mean expected heterozygosity (H_e) for each pig population.

Table 2. Number of Pigs Identified with Zoonotic Parasites Detected in This Study for Each Genetically Discrete Feral Pig Population

	Zoonotic parasites				
Population	*Giardia*	*Entamoeba*	*Balantidium*	*Cryptosporidium*	*Chilomastix*
Perth Hills (Northern catchments)	2	1	0	0	0
Serpentine	3	4	9	0	3
Dandalup	0	4	0	1	0
Collie	0	0	1	0	0

(Cox, 1998; Solaymani-Mohammadi et al., 2004). A number of principally pig-specific parasites were found in specimens from all catchment areas, particularly *Metastrongylus* spp., and less commonly strongyles, *Eimeria* spp. and *Ascaris suum*.

All of the pigs sampled in metropolitan water catchment areas formed part of one of three large separate feral pig populations. These were designated as Northern Catchments, Serpentine, and Dandalup (Fig. 1, Table 2). Each population was found to be genetically unique, with a few exemptions. This indicates that gene flow is high along these watercourses compared with the relatively low level of gene flow observed over similar distances between these river systems (see Hampton, 2004a). The detection of recent illegal release of pigs (Spencer and Hampton, 2005) and the presence of feral pigs around the fringes of the water catchment dams could represent a major public health threat. Interestingly, 9 of 10 *Balantidium* specimens were from the 3 catchment areas comprising the Serpentine population. The other case was in Samson Brook (Collie population), while the Dandalup and Northern Catchments populations appear to be totally free of this parasite. This finding supports the genetic data that indicate the three catchment areas constituting the Serpentine population are isolated from the surrounding catchment areas. Also, three of the five cases with *Giardia* were reported from the Serpentine population. The other two *Giardia* cases were from Northern Catchments pigs. Fifteen animals

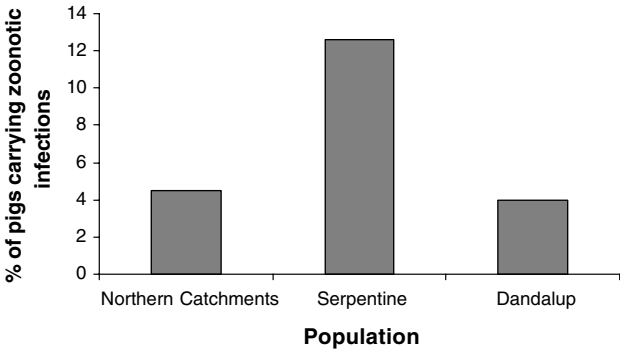

Figure 2. Percentage of sampled feral pigs carrying potentially zoonotic waterborne pathogens from the three northern, genetically distinct populations. Samples are grouped according to pigs caught from the Northern (n = 75), Serpentine (n = 116), and Dandalup (n = 149) catchments illustrated in Figure 1.

(13%) from the Serpentine population were carrying at least 1 of the 4 potentially zoonotic parasites, while only 3 pigs (4%) from the Northern population and 6 pigs (4%) from the Dandalup population were carrying these same pathogens (Fig. 2). Dandalup appears to be the "cleanest" population, with no *Giardia* or *Balantidium* cases identified from the 149 individuals tested. However, the sole suspected case of *Cryptosporidium* did come from the Dandalup population.

Previous studies (Atwill et al., 1997) have suggested that *Cryptosporidium* prevalence is much higher in high-density feral pig populations, and that the infection is not maintained in low-density feral pig populations. This finding is consistent with the fact that the sole case of *Cryptosporidium* identified by this study came from the catchment area with the highest recent catch rate, indicating a relatively high feral pig density. This trend makes reduction of feral pig abundance in high-density catchment areas even more important to reduce the risk of waterborne feral pig pathogens being introduced to reservoirs. The study by Atwill et al. (1997) also showed that *Cryptosporidium* is most common in pigs of less than 6 months of age. As the traps used in the present study had a mesh of sufficient size to allow these very small pigs to escape, this age group is underrepresented in the samples analyzed.

The genetic results are indicative of a low rate of social contact between pigs, and when this is considered in conjunction with the very low numbers of pigs that move between populations (Hampton et al., 2004b), the risk of any introduced directly transmitted disease spreading throughout the region by feral pigs is very low. Given the habit of pigs to wallow in mud and shallow water, and the fact that most feral pigs in a catchment area have daily contact with either feeder creeks or the reservoir itself (especially in summer), the chances of potential pathogens reaching the main water supply may be high.

The confinement of the majority of *B. coli* cases to particular areas suggests that some catchments represent a greater contamination threat. Similar findings were reported in the geographic prevalence of *Giardia* and *Cryptosporidium* in drinking water in Canada (Wallis et al., 1996). It is clear that the incorporation of genetic, spatial, and biological prevalence studies in relation to zoonotic threats will have consequences for new types of intervention and ultimately achieve opportunities for environmental and human health protection not presently undertaken (Parkes et al., 2003).

In conclusion, our results suggest that feral pigs represent a potential risk to drinking water supplies in our study catchments. Further study is required to determine the fate and possible transport of pathogens excreted in the catchments, and whether feral pig populations pose a significant public health risk in this regard. Moreover, our results from genetic analysis of pigs indicate a high level of population substructuring along distinct infection prevalence rates among population units. This substructuring, resulting from spacing behavior, apparently influences cross-population pathogen transmission, thus possibly pathogen persistence. Combining pathogen detection and host population genetic analysis as demonstrated here may be particularly useful in targeting host population control measures as a means of managing waterborne disease risk.

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