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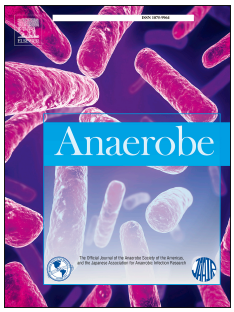

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Persistence of *Clostridium difficile* RT 237 infection in a Western Australian piggery

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ABSTRACT

Clostridium difficile is commonly associated with healthcare-related infections in humans, and is an emerging pathogen in food animal species. There is potential for transmission of *C. difficile* from animals or animal products to humans. This study aimed to determine if *C. difficile* RT 237 had persisted in a Western Australian piggery or if there had been a temporal change in *C. difficile* diversity. *C. difficile* carriage in litters with and without diarrhea was investigated, as was the acquisition of *C. difficile* over time using cohort surveys. Rectal swabs were obtained from piglets aged 1-10 days to determine prevalence of *C. difficile* carriage and samples were obtained from 20 piglets on days 1, 7, 13, 20, and 42 of life to determine duration of shedding. Isolation of *C. difficile* from feces was achieved by selective enrichment culture. All isolates were characterized by standard molecular typing. Antimicrobial susceptibility testing was performed on selected isolates (n=29). Diarrheic piglets were more likely to shed *C. difficile* than the non-diseased (p=0.0124, χ^2). In the cohort study, *C. difficile* was isolated from 40% samples on day 1, 50% on day 7, 20% on day 13, and 0% on days 20 and 42. All isolates were RT 237 and no antimicrobial resistance was detected. The decline of shedding of *C. difficile* to zero has public health implications because slaughter age pigs have a low likelihood of spreading *C. difficile* to consumers via pig meat.

Keywords: *Clostridium difficile*; Epidemiology; *C. difficile* shedding; Neonatal pigs; Diarrhea.

1. Introduction

Clostridium difficile is a Gram positive, strictly anaerobic, spore forming bacterium commonly associated with healthcare-related infections (*C. difficile* infection, CDI) and responsible for 20% of all antibiotic-associated diarrhea and colitis in humans [1]. *C. difficile* produces two toxins, A (an enterotoxin) and B (a cytotoxin), which are the main virulence factors [2]. Some strains produce a third unrelated toxin, an ADP-ribosyltransferase (binary toxin), the exact role for which is yet to be determined - although some studies suggest that it contributes to disease severity [3].

C. difficile is an emerging pathogen in food animals that has been recovered from the gastrointestinal tracts of multiple production animal species [2]. Piglets are colonized soon after birth, generally within 1-7 days [4, 5]. Colonization is most common in younger piglets, with older pigs being culture-negative by 2 months of age [6]. Like other porcine enteric pathogens, *C. difficile* has been isolated from both non-diseased piglets and those with clinical diarrhea [2, 6, 7]. Toxins A and B, or just B alone, have been detected in both diarrheic and non-diarrheic piglets [7]. This suggests that several other factors are important in the manifestation of disease [3]. Infected piglets may succumb to diarrhea and mortality rates of up to 50% have been reported in some outbreaks. Those that survive can be underweight by 10%-15%, which can delay weaning [8] and may affect profitability of pig farms.

Outbreaks of CDI in pig herds, and also humans, have been reported frequently since the early 2000s [9, 10]. Of particular interest was the rise in incidence of a so-called “hypervirulent” strain PCR ribotype (RT) 027 (also known as NAP1/BI), initially in North America and later in Europe [10]. This coincided with CDI outbreaks in animals, although RT 078 was reported as the predominant strain colonizing cattle and pigs [3, 11, 12]. Increasingly, studies have shown

genetic overlap between animal and human strains of *C. difficile* RT 078 [13, 14], supporting the theory of zoonotic transmission.

In 2009, a farrow to finish commercial piggery in Western Australian was experiencing idiopathic diarrhea in up to 80% of neonatal pigs with mortality in the range 11-14%. The affected piglets had early-onset of diarrhea which was yellow, non-hemorrhagic, and pasty to watery. Untreated piglets had ill-thrift, became anorexic and dehydrated, and some died. Apparently healthy piglets (1-3 days old) were prophylactically treated with amoxicillin or penicillin. A cross-sectional study in the piggery found a *C. difficile* prevalence of 62% (114/185) in 5-7 day-old piglets [15]. In that study, molecular typing revealed all isolates of *C. difficile* recovered were an unusual RT 237, toxinotype XXXI (*tcdA*$^-$, *tcdB*$^+$), binary toxin positive *(cdtA/B*$^+$) strain. Few studies have described the epidemiology of infections in livestock with RTs of *C. difficile* other than RT 078 [12, 15-17]. This study aimed to determine if *C. difficile* RT 237 had persisted in the same piggery or whether there had been a temporal change in *C. difficile* diversity. *C. difficile* carriage in litters with and without diarrhea was investigated, as was the acquisition of *C. difficile* over time.

2. Materials and Methods

2.1 Study design

The study designs used to address the aims were single cross-sectional and prospective cohort studies, with sampling conducted from October to December 2014. The piggery was located across two sites. The farrow-to-wean site had two holdings separated by a fence, with approximately 5000 sows; holding “A” consisted of older breeding sows (parity>1) and holding “B” consisted of gilts. Holding “C” was the finishing site some 20 km away. The sample size for the cross-sectional study was determined using Fleiss methods with a continued correction factor

92 [18]. We estimated that 47.4% of non-diarrheic piglets were shedding *C. difficile* and 92.8% of
93 diarrheic piglets were exposed. The ratio of non-exposed piglets to exposed piglets was assumed
94 to be 0.5, and with an odds ratio of 14, and a power of 80% to detect the difference if it existed, a
95 sample size of 43 piglets was selected. Fresh fecal samples were collected via rectal swabs from
96 4 or 5 piglets randomly selected from each of 9 litters aged 1-10 days.

97 For the cohort study, we estimated a difference of 27% prevalence of *C. difficile* shedding
98 between 1 day-old (77%) and 42 day-old piglets (50%) based on earlier studies [15]. Using a two
99 tail Z-test for logistic regression, with α of 0.05% and power of 80%, we determined that a total
100 sample of 88 piglets was required. To account for possible loss to follow up of 12%, 12 piglets
101 were added to the sample to make a total of 100. Fecal samples (n=20) were randomly obtained
102 from 5 piglets from each of 4 litters as described above on days 1, 7, 13 and 20, at the farrow-to-
103 wean holding and on day 42 at the finishing site. One day before weaning, 20 piglets were ear
104 tagged to allow follow-up at the finishing site. Among the four litters studied, two had 10 piglets
105 each and the others had 14 piglets each. All swabs were transported in Amies transport medium
106 with charcoal (Thermo Fisher Scientific, Waltham, MA, USA) in a cooler box at 4^{o}C to The
107 University of Western Australia, School of Pathology and Laboratory Medicine, for processing
108 within 24 h.

109 This piggery had a two stage in-series anaerobic pond system for treatment of effluent. The
110 primary aerobic pond has an inlet design to facilitate easier desludging of the pond. After moving
111 through the primary pond, effluent moved to a secondary pond which allowed reuse and storage.
112 No chemical disinfection was applied to the water. Therefore, an additional four 30 ml specimen
113 jars (Techno-Plas Pty Ltd, St Marys, Australia) of treated water held for under-pen flushing in
114 storage tanks located adjacent to the farrowing shed, four 30 ml effluent samples from a drainage

channel leading to the aerobic pond, and six shed floor swabs transported in Amies transport medium with charcoal were obtained from holding "A". The six floor swabs were obtained by directly swabbing the wet floor from six pens.

Additional data were collected such as the health status of the piglets, age, litter size, mortality, parity of sow and farrowing date. A piglet was considered diarrheic at the sampling time using the following criteria: i) had yellow, non-hemorrhagic, and pasty to watery feces and ii) any piglet painted red at the dorsum by personnel on the basis of diarrhea being observed, and that had a perineum soiled with watery feces. A litter was classified as diarrheic if one or more piglets had diarrhea at the time of sampling.

2.2 Isolation of *C. difficile*

C. difficile was isolated as previously described, with minor modifications [19]. Briefly, the swabs were cultured directly on ChromID™ agar (bioMérieux, Marcy l'Etoile, France) and in an enrichment broth containing cefoxitin, cycloserine and gentamicin. Following alcohol shock when 1 ml of 48 h broth culture was mixed with 1ml anhydrous ethanol (96%) and left for 1 h, 0.01 ml of mixture was cultured on ChromID™ agar. Effluent and treated water samples (10 ul) were cultured directly on ChromID™ agar or following broth enrichment. An aliquot of 1 ml of either effluent or treated water was transferred to the enrichment broth and processed similarly to feces.

All cultures were incubated anaerobically (A35 anaerobic chamber, Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) at 37°C, with an atmospheric gas composition of 80% N_2, 10% CO_2 and 10% H_2. Two to three probable *C. difficile* colonies on ChromID™ agar were cultivated on blood agar and identified on the basis of their characteristic chartreuse

fluorescence detected with UV light (~360nm wavelength), colonial morphological characteristics (ground glass appearance) and horse dung odor. Identification of uncertain isolates was achieved by Gram staining and detection of L-proline aminopeptidase (Remel Inc., Lenexa, KS, USA).

2.3 Molecular characterization

All isolates were characterized by PCR to determine the presence of toxin A (*tcdA*), B (*tcdB*), and binary toxin (*cdtA* and *cdtB*) genes and changes in the repetitive region of the toxin A gene [20]. PCR ribotyping was performed on strains as described elsewhere [21]. RTs were identified by comparing their banding patterns with those in our reference library of animal and human *C. difficile* strains, consisting of a collection of 50 Anaerobe Reference Laboratory (ARL, Cardiff, UK) ribotypes that included 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and the most prevalent PCR ribotypes currently circulating in Australia [B. Elliott, T. V. Riley, unpublished data].

2.4 Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for 14 antimicrobials were determined for a selection of isolates using the agar incorporation method as described by the Clinical and Laboratory Standards Institute (CLSI, M11-A7) [22]. A combination of CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints was used if available [23, 24]. The quality control strains used were *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *C. difficile* ATCC 700057 and *Eubacterium lentum* ATCC 43055.

2.5 Statistical analysis

159 The Chi-squared test was used to evaluate the association between isolation of *C. difficile*
160 and diarrhea in the cross-sectional study. *C. difficile* shedding over time was evaluated by the
161 generalized estimating equations (GEEs) for longitudinal data collected in clusters that are
162 repeated measures. The outcome variable was considered as binary (presence or absence of *C.*
163 *difficile* per sample) and fixed effects models were employed in GEEs to adjust for the response
164 variable from within clusters (litters) as well as over time (6 weeks). In fitting the data to the
165 model we used the independent working correlation structure as this implies that the within-litter
166 correlation between all sampling was equal to zero. GEEs have been shown to be robust even
167 when there is an error in specifying the working correlation structure [25]. All associations with
168 a p value ≤ 0.05 were considered significant. All analyses were performed in Epi-Info^{TM} 7.1.4.0
169 statistical software from the Centers for Disease Control and Prevention (CDC) and R version
170 3.2.2.

171 **Animal ethics committee approval.** This study was approved by The University of
172 Western Australia Animal Ethics Committee (reference number RA/3/500/75).

173 **3. Results**

174 3.1. *C. difficile* carriage in piglets

175 *C. difficile* was isolated from 19/43 (44.2%, 95% CI 29.3%-59.1%) fecal swabs by direct
176 culture and 29/43 (67.4%, 95% CI 53.39- 81.41) with enrichment media from holding "A".
177 Enrichment culture was significantly more sensitive than direct culture (p=0.0002, χ^2). Of the
178 diarrheic piglets, 20 of 24 (83.3%) were *C. difficile* culture positive compared to 9 of 19 (47.4 %)
179 non-diarrheic piglets (p = 0.0124, χ^2). *C. difficile* was isolated from piglets in 7 out of 9 pens
180 (77.9%).

A total of 13/106 (12.3%) piglets died across the nine litters sampled in the cross-sectional study, however, the association between *C. difficile* positive status and mortality was not significant (p=0.74). There were seven litters with and two without diarrhea and a total of 24 out of 43 diarrheic piglets. The comparison between parity and *C. difficile* positive status of piglets was made between parity 3 (referent) and combined piglets from sows with parity 4, 5 and 6 because of sparse data. *C. difficile* distribution in piglets by parity of sow was parity 3 (13/19; 68.4%), parity 4 (7/10; 70%), parity 5 (5/9; 55.6%), and parity 6 (4/5; 80%). All *C. difficile* isolates from piglets were RT 237.

3.2. The prospective cohort study

C. difficile was isolated from 8/20 fecal samples (40%) on day 1, 10/20 (50%) on day 7, 4/20 (20%) on day 13, 0/20 (0%) on day 20, and 0/20 (0%) on day 42 (Table 1). The multivariate model evaluated the following variables: age of piglets, litter size, mortality and diarrhea (Table 1). There was no significant difference between *C. difficile* shedding on day 1 versus day 7 (p= 0.10), nor day 1 versus day 13 (p= 0.10). However, there was a significant difference in *C. difficile* shedding between 1 day-old piglets and piglets at 20 and 42 days of age (p<0.000). The regression coefficients were positively associated with *C. difficile* shedding on day 7 but were strongest and negatively (inversely) associated with shedding on day 13 to day 42 (Table 1). The risk of shedding *C. difficile* in the feces by piglets significantly declined from day 13 onwards, as the regression coefficients were negative (inverse) (Table 1). The overall prevalence of *C. difficile* was 22% (22/100). There was a total of 48 piglets from the four litters studied. This means that 42% of piglets were sampled at each time point indicating that each piglet had 42% chance of being sampled every week. *C. difficile* was isolated at least once from all study litters 100% (4/4).

C. difficile was not isolated from piglets aged 20 days and 42 days (n=20) (Table 1). There was a total of 36/100 cases (36%) of diarrhea among the sampled piglets. The cases of diarrhea in piglets per sampling time were as follows: day 1 (8/20; 40%), day 7 (6/20; 30%), day 13 (11/20; 55%), day 20 (9/20; 45%) and day 42 (2/20; 10%). However, the association between *C. difficile* positive status and diarrhea for all cases was not significant (p= 0.67).

Nine piglets from four litters died in this study, giving a 9% mortality rate. Seven of the piglets were from diarrheic pens where *C. difficile* was identified, while two were from non-diseased but *C. difficile* positive pens. The regression coefficient for mortality was positively associated with *C. difficile* shedding (p=0.001) (Table 1).

The toxin B gene (*tcdB*) but not *tcdA* was detected by PCR in all *C. difficile* isolates from the 22 infected piglets, including both diarrheic and non-diarrheic animals. Binary toxin genes (*cdtA* and *cdtB*) also were detected in all isolates and all were RT 237.

3.3. Environmental samples

The effluent samples (n=4) obtained from a drainage channel before the two-stage treatment ponds were all positive for *C. difficile* by enrichment culture. Additionally, two of the four samples of treated water collected from the farrowing sheds were positive. Furthermore, four of the six floor swab samples collected from some of the pens of diarrheic and non-diarrheic litters were positive (67%). All environmental isolates were RT 237.

3.4. Antimicrobial susceptibility profiles

MICs for 14 antimicrobials were determined for 29 isolates sourced from the cross-sectional study (Table 2). Despite the probability that these isolates were clonal, there were some small variations in susceptibility; however, all were susceptible to the antimicrobials for which

breakpoints were available. There are no CLSI and EUCAST breakpoints available for the following antimicrobials; gentamicin had MIC range (32-64 mg/L), spectinomycin (128 mg/L), tobramycin (32-128mg/L), and trimethoprim (32-64mg/L).

4. Discussion

This study aimed to determine if *C. difficile* RT 237 had persisted in the Western Australian piggery that we investigated in 2009 [15]. *C. difficile* RT 237 was found again and the prevalence in the cross-sectional study (67.4%) was similar to the earlier study (62%) [15], and the same as a national prevalence study conducted recently in 21 Australian piggeries (67%) [16]. In the Australian national survey RT 014, a strain commonly reported in human hospital settings [26, 27], was the most prevalent RT found (36/154; 23%). Overall these prevalence results are consistent with findings in studies from Europe [17] and North America [11]. However, the reasons for continuing predominance of RT 237 in this piggery are unclear. One possible explanation is that the piggery generates its own replacement breeding stock and this could have prevented introduction of new *C. difficile* strains from other piggeries. Our findings suggest that new strains of *C. difficile* are not commonly introduced from other sources such as rodents or birds on this piggery. An important factor could be the geographical location of the piggery both within the State of Western Australia, and within Australia generally where there is a large expanse of desert and great distances separating eastern and western Australia.

The prevalence in the cross-sectional study on holding "A" was 67.4% in piglets aged 1-10 days, and the overall prevalence of *C. difficile* from the cohort study was 22% (22/100). There was a gradual decline in *C. difficile* shedding in feces with increasing age of piglets in the cohort study on holdings "B" and "C". These findings are in agreement with similar studies from

249 elsewhere [4, 28] and with other cross-sectional studies [6, 15- 17, 29] which reported a lower
250 prevalence of *C. difficile* in older (>14 days) piglets than in younger piglets. Álvarez-Pérez *et al*.
251 [6] reported a 26% prevalence of *C. difficile* in piglets aged 1-7 days in Spain but zero
252 prevalence in pigs aged 1 to 2 months, while a study conducted in an integrated swine production
253 system in the USA found that fecal shedding of *C. difficile* was 50% in suckling piglets, 6.5% in
254 weaner pigs (3-10 weeks old) and 3.9% in both fattening pigs (up to 22 weeks) and adult
255 breeding boars and sows [29]. Another longitudinal study undertaken in Canada found a *C.*
256 *difficile* prevalence of 74% (day 2), 55% (day 7), 40% (day 30), 23% (day 44) and 3.7% (day 62)
257 [4]. These findings support the hypothesis that *C. difficile* colonization declines with increasing
258 age, possibly due to interference from developing components of the normal intestinal
259 microbiota in a phenomenon referred to as "colonization resistance" [30].

260 A high prevalence of *C. difficile* in slaughter age pigs could pose a risk of foodborne
261 infection to humans through consumption of contaminated meat. The current study did not
262 examine slaughter age pigs, but the overall prevalence found in younger pigs was 22% (22/100),
263 lower than that reported in Canada (96%) [4] and in the Netherlands (100%) [5], but similar to
264 that reported in Spain (25.6%) [28], although the RTs detected were different. Álvarez -Perez *et*
265 *al*, [28] found a peak prevalence on day 15 (85%) compared to day 7 (50%; 10/20), but they
266 sampled from the same piglets over time up to day 50 as opposed to sampling a subset of the
267 same litters over time. The decline in *C. difficile* shedding to zero by day 20 was earlier than
268 reported in other studies [4, 28] where *C. difficile* shedding continued up to day 50. Weese and
269 colleagues [31] reported a farm level *C. difficile* prevalence of 6.5% (30/346) in slaughter age
270 pigs in Canada. In that study, various strains of *C. difficile* were detected, but RT 078 was the
271 predominant strain on farms, with a prevalence of 67% [31]. Many other studies have

documented the presence of *C. difficile* in meat products such as retail beef, pork and turkey [32, 33]. The fact that *C. difficile* was not isolated in older pigs (6 weeks old) in the present study suggests that slaughter age pigs at this piggery are unlikely to pose a risk for human infection. However, there is a need to carry out further studies at local piggeries with different circulating RTs and in abattoir environments to be able to exclude local meat products as a source of *C. difficile.*

The contaminated farm environment may provide a source of *C. difficile* for human infection. *C. difficile* can be dispersed by wildlife [34], vermin (mice and flies on a piggery) [35], wind [36], and manure [33]. RT 078, a well-established animal pathogen, has increasingly been isolated from humans, particularly those living near pig farms in Europe [13, 37]. Knetsch *et al* reported indistinguishable strains of *C. difficile* RT 078 in pig farmers and pigs by whole genome sequence techniques [11]. In the present study, RT 237 was detected from the floor, treated water, and also from effluent from a drainage channel before the two–stage treatment pond at the piggery. Similarly, Squire and colleagues isolated *C. difficile* RT 237 from treated pig effluent planned for use in cleaning the pig sheds [38]. However, RT 237 has been detected rarely in clinical specimens obtained from human patients in Western Australia [26, 39], suggesting, perhaps, that it does not adapt well to a human host.

At the study piggery, a sporicidal disinfectant (sodium hypochlorite) has been used in pig sheds for the last few years. An explanation for detection of *C. difficile* from pen floor and waste-water is not obvious although suboptimal concentration of the disinfectant used cannot be ruled out. *C. difficile* spores can persist in the environment for a long time, therefore additional control measures such as providing education to all working staff at the farm could further reduce the

incidence of CDI. Overall, these findings suggest that sporicidal disinfectants in pig sheds analogous to hospital environments may reduce piglet infections [40].

All the *C. difficile* isolates sourced from the cross-sectional study had similar susceptiblities to a panel of antimicrobials, with no resistance detected (Table 2). This finding was expected because all isolates were most likely clonal. In an earlier smaller study of RT 237 isolates from the same piggery no resistance was detected [41]. In contrast, Peláez *et al*. [42] reported a 9% prevalence of metronidazole resistance (MIC>256 mg/ml) and nearly 50% multi-drug resistance in *C. difficile* in swine herds in Spain. In general, there is a paucity of information on antimicrobial susceptibility of *C. difficile* in livestock.

4.1 Conclusions

RT 237 has persisted for at least 5 years and remains the predominant strain of *C. difficile* in piglets on a piggery in Western Australia. This unusual RT has been detected in human patients in Australia but not in high numbers. The decline of *C. difficile* shedding to zero by day 20 suggests that slaughter age pigs are unlikely to be greatly contaminated with *C. difficile* in this piggery. Further research is warranted to determine the sources of the persisting RT 237 on the piggery, and to reduce contamination levels in the piggery environments to limit piglet and potentially human exposure.

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Conflict of interest

316 The authors have no conflict of interest to declare.

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440 **Table 1** Diarrhea and *C. difficile* shedding over time by piglets in relation to their age.

Variable	[a] *C. difficile* positive			*C. difficile* negative			GEEs Regression		
	[b]D^+	[c]D^-	[d]Total	D^+	D^-	[d]Total	Coefficients	Std.error	P-value
Intercept							1.9218	2.40	
Day 1	2	6	8	6	6	12	Referent		
Day 7	3	7	10	3	7	10	1.511	1.10	0.10
Day 13	4	0	4	7	9	16	-1.1701	0.82	0.10
Day 20	0	0	0	9	11	20	-43.15	1.09	0.000
Day 42	0	0	0	2	18	20	-43.15	1.14	0.000
Litter size							-0.28	0.17	0.05
Mortality	7	2	9	0	0	0	0.48	0.17	0.001

441

442 Note.[a] *C. difficile* test, [b]D^+ diarrheic, [c]D^- non-diarrheic, [d]Total

443

444 **Table 2** Minimal inhibitory concentration (MIC) range and percentage distribution for RT 237 isolates (*n*=29) against a panel of 14

445 antimicrobial agents.

Agent	MIC range [mg/L]	Clinical breakpoints			Percentage distribution (%)		
		S	I	R	S	I	R
Vancomycin	1	≤2	*NR*	≥2	100	0	0
Metronidazole	0.25 - 1	≤8	16	≥32	100	0	0
Clindamycin	0.25 - 4	≤2	4	≥8	65.5	34.5	0
Erythromycin	0.25 - 0.5	*NR*	*NR*	>8	*NR*	*NR*	0
Amox-clavulanate	0.12 - 0.25	≤4	8	≥16	100	0	0
Ceftriaxone	8	≤16	32	≥64	100	0	0
Moxifloxacin	1	≤2	4	≥8	100	0	0
Meropenem	0.25 - 2	≤4	8	≥16	100	0	0
Tetracycline	0.12	≤4	8	≥16	100	0	0
Piperacillin/tazobactam	2 - 4	≤32	64	≥128	100	0	0

446

447 Note. The susceptible (S), intermediate (I), and resistance (R) interpretive values when available were obtained from CLSI or

448 EUCAST (vancomycin only). If breakpoints were not available from CLSI and EUCAST then a no range (NR) initial was written.

449

Highlights

- We describe the epidemiology of *Clostridium difficile* in a Western Australia piggery
- We have demonstrated a relationship between age of piglets and fecal shedding of *C. difficile*.
- We have shown that ribotype 237 has persisted in one piggery in Western Australia for over six years.