PRODUCTION ANIMALS

Testing the efficacy of kitasamycin for use in the control and treatment of swine dysentery in experimentally infected pigs

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Background Swine dysentery (SD) caused by *Brachyspira hyodysenteriae* is an important disease in Australia.

Aim The aim of this study is to evaluate the macrolide antibiotic kitasamycin for use in SD control.

Methods The minimum inhibitory concentrations (MICs) of kitasamycin, tylosin and lincomycin for 32 Australian isolates of *B. hyodysenteriae* were evaluated. Mutations in the 23S rRNA gene were examined. Isolate '13' with a low kitasamycin MIC was used to challenge weaner pigs. Sixty pigs were housed in 20 pens each containing three pigs: pigs in four pens received 2 kg/tonne of a product containing kitasamycin (3.1% active) prophylactically in their food starting 4 days before *B. hyodysenteriae* challenge (group 1); pigs in four pens were challenged and received the same dose therapeutically once one pig in a pen showed diarrhoea (group 2); four pens were challenged and received 4 kg/tonne of the product therapeutically (group 3); four pens were challenged but not medicated (group 4); two pens were unmedicated and unchallenged (group 5) and two pens received 2 kg/tonne and were unchallenged (group 6). Pigs were monitored for *B. hyodysenteriae* excretion and disease.

Results Macrolide resistance was widespread, and mutations in the 23S rRNA gene were identified in 23 isolates. Four isolates with kitasamycin MICs < 5 µg/mL were considered susceptible. Following experimental challenge, 10 of 12 unmedicated pigs developed SD. No pigs receiving kitasamycin prophylactical or therapeutically developed SD. Medicated pigs shed low numbers of *B. hyodysenteriae* in their faeces.

Conclusions Kitasamycin can help control SD in pigs infected with susceptible isolates of *B. hyodysenteriae*.

Keywords antimicrobial resistance; *Brachyspira hyodysenteriae*; kitasamycin; macrolide; swine dysentery

Abbreviations ECOFF, epidemiological cut-off value; MIC, minimum inhibitory concentration; ppm, parts per million; pm, postmortem; SD, swine dysentery; SNP, single nucleotide polymorphism

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Swine dysentery (SD) is a potentially severe mucohaemorrhagic colitis of pigs that occurs mainly in the growing and finishing phases of production.[1] SD most commonly results from infection caused by the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*, although the related species *Brachyspira hampsonii* and *Brachyspira suanatina* may also cause SD in some countries.[2,3] The latter two species have not been identified in Australia, but *B. hyodysenteriae* is present in up to a third of Australian herds – including in herds where clinical SD has not been reported.[4]

No effective commercial vaccines for SD are currently available, and control in infected herds is heavily reliant on prophylactic or therapeutic use of antimicrobials. The main classes of antimicrobials to which *B. hyodysenteriae* has been reported to be susceptible include macrolides (tylosin and tylvalosin), lincosamides (lincomycin), pleuromutilins (tiamulin and valnemulin), imidazoles (dimetridazole), quinoxalines (carbadox), streptogramins (virginiamycin) and ionophores (salinomycin and monensin). Of these, only tylosin, lincomycin and tiamulin remain registered for use in SD control in Australia. Furthermore, reduced susceptibilities to one or more of these antimicrobials increasingly have been reported among Australian isolates of *B. hyodysenteriae*,[4–6] including identification of virulent isolates that are resistant to all the three available antimicrobials.[4] Multidrug resistant isolates of *B. hyodysenteriae* have also been reported from the Czech Republic,[7] the Netherlands,[8] and Italy,[9] thus emphasising the global nature of this problem. The existence of resistant isolates is causing serious difficulties for SD control and eradication, and consequently other antimicrobials and alternative treatments are being sought to achieve this.

In Australia, a product (Trubin L-50 Leucomycin A5 [3.1% kitasamycin active], Bayer, Leverkusen, Germany) containing the macrolide antibiotic kitasamycin has been available for use as a growth promotant for pigs, with an inclusion rate of 1 to 2 kg per tonne of feed, equivalent to 0.031 to 0.062 g/kg of kitasamycin, or 31 to 62 parts per million (ppm). There are legitimate concerns that the use of antimicrobial drugs at low concentrations for long periods will help to select for bacterial strains with reduced susceptibility to the antimicrobial and to related antimicrobials, and hence this product ceased to be registered for growth promotion purposes in Australia in April 2019. Although kitasamycin is an early generation macrolide, it still may have potential for treating cases of SD where the causal spirochaete is sensitive to kitasamycin. The purpose of this study was to determine whether kitasamycin could be used to help control SD in Australia, and potentially be registered for use as a therapeutic drug for this purpose.

Materials and methods

Approvals

The experimental animal study was undertaken with the approval of the Murdoch University Animal Ethics Committee (approval number R3009/17).

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B. hyodysenteriae *isolates*

Thirty-one isolates of *B. hyodysenteriae* recovered from different Australian pig herds between 2014 and 2018 were selected for use, together with Australian reference strain WA1. The isolates had been recovered from faecal samples submitted to the diagnostic laboratory at Murdoch University, and 28 of them had been subjected to whole genomic sequencing, as previously described.[10] Reference strain WA1 was isolated from a pig in Western Australia in the 1980s, and was the first strain of *B. hyodysenteriae* from which a whole genome sequence was obtained.[11]

Testing in vitro susceptibility to kitasamycin and other antimicrobials

The 31 *B. hyodysenteriae* isolates and strain WA1 were tested for their susceptibility to kitasamycin, tylosin and lincomycin. The latter two antimicrobials were tested, because macrolides and lincomycin may share mutational changes at binding sites on the 23S rRNA gene that are associated with reduced susceptibility to these drugs. The isolates were subcultured to Trypticase Soy agar (TSA) plates (BBL, Franklin Lakes, NJ, USA) containing 5% (vol/vol) defibrinated ovine blood and incubated for 5 days at 37°C in a jar with an anaerobic atmosphere generated using an AnaeroGenTM 2.5L Sachet (Oxoid, Basingstoke, Hampshire, UK). The surface growth was harvested and suspended in 2 mL sterile phosphate buffered saline (PBS), then transferring into a microfuge tube and centrifuged at 5000 *g*. The pellet was resuspended in sterile PBS; the cells were counted using a haemocytometer and then diluted to a density of 10^6 cells per mL.

Antimicrobial susceptibility was assessed using the agar-dilution method. Test plates consisted of TSA containing 5% defibrinated ovine blood and the appropriate antibiotic concentration, while control plates did not include antibiotics. The isolates were tested for susceptibility to varying concentrations of kitasamycin (0.675, 1.25, 2.5, 5.0, 10, 20, 40, 80 and 160 µg/mL), tylosin (1, 4, 25, 50 and 100 µg/mL) and lincomycin (2, 4, 16, 36 and 72 µg/mL).

For each isolate, 10^5 cells were drop-inoculated onto the control and sensitivity plates. Each isolate was tested in duplicate, and *B. hyodysenteriae* control strain WA1 was included in each batch of tests. Growth of the isolates on the control and sensitivity plates was checked visually after 5 days of incubation. Zones of haemolysis around the growth on the control plates were determined, and isolates were recorded as being susceptible to the antimicrobial concentration in the test plates if no such zones were observed. Surface growth was scraped off the plate and examined under a phase contrast microscope to confirm purity and the endpoint. The first sensitive growth zone and the last resistant growth zone were checked for spirochaetes by phase-contrast microscopy. The minimum inhibitory concentration (MIC) of the antimicrobial was reported as the lowest concentration that inhibited growth.

Estimating the epidemiological cut-off value for kitasamycin

The ECOFFinder spreadsheet calculator from the Clinical and Laboratory Standards Institute (https://clsi.org/meetings/microbiology/ecoffinder/) was used to estimate the epidemiological cut-off values (ECOFFs) for kitasamycin MICs, thus defining the wild-type and resistant populations of the isolates with respect to kitasamycin.

23S rRNA gene sequence analysis

The 23S rRNA gene sequences of 28 of the isolates and strain WA1 were examined for single nucleotide polymorphisms (SNPs) in the nucleotide positions homologous with positions 2058 and 2059 of the *Escherichia coli* 23S rRNA gene which have been previously associated with resistance to macrolides and lincosamides in *Brachyspira*.[12,13] SNPs at other positions were also examined. Sequence data were not available for the other three isolates.

Animal trial

Pigs and diet. Sixty castrated male weaner pigs of approximately 9 kg body weight were purchased from a Western Australian commercial piggery that was free of SD based an absence of clinical signs and negative results from regular screening by selective culture and PCR testing for *B. hyodysenteriae*. On arrival at Murdoch University, the pigs were weighed, ear-tagged and randomly assigned to be housed in groups of three per pen in three rooms of an isolation animal house. The temperature was set at 26°C for the first 2 weeks, and the pigs were offered water and feed ad libitum. They were fed on an unmedicated commercial pelleted weaner feed for the first 10 days and then transferred to an unmedicated commercial pelleted grower feed containing wheat, lupins and meat meal. The grower feed for groups 1, 2, 3 and 6 was medicated with the commercial kitasamycin product as dictated by the experimental design.

Table 1. Experimental groups of pigs and their treatments

Group	Kitasamycin ppm (regimen[a])	*Brachyspira hyodysenteriae* challenge	Number of pens[b]	Rooms[c]	Number of pigs
1	62 (prophylactic)	+	4	A, B	12
2	62 (therapeutic)	+	4	A, B	12
3	124 (therapeutic)	+	4	A, B	12
4	Nil	+ (+ve control)	4	A, B	12
5	Nil	– (–ve control)	2	C	6
6	62 (prophylactic)	– (–ve prophylaxis control)	2	C	6

[a]Prophylactic indicates that the diet containing kitasamycin was added from 4 days before the first day of experimental challenge, and thereafter. Therapeutic indicates that the diet containing kitasamycin was given to pigs in a pen from the first day that one pig in that pen developed diarrhoea (faecal score 3). Medication was continued until the end of the experiment. [b]Three pigs per pen. [c]For groups 1–4, half the pens were in room A and half in room B.

Table 2. Faecal consistency score

Faecal consistency	Faecal score
Normal, formed	0
Soft	1
Porridge-like	2
Diarrhoea	3
Diarrhoea containing blood and/or mucus	4

Medication was achieved by top-dressing with the powdered product. Mixing was done in 3 kg batches of pellets/pen, with the product being thoroughly manually mixed with the pellets. Pellets were used rather than meal, as this reduced wastage and allowed closer monitoring of the consumption of the product. Troughs were checked three times a day, and when approximately half the feed in a trough had been eaten, the remainder was remixed manually to ensure that any product that had settled to the bottom of the trough was redistributed. All feed was topped up regularly so that it was available ad libitum.

Experimental design. Six experimental groups were used, with the animals assigned to the groups being penned in subgroups of three per pen in three rooms (Table 1). Pigs in group 1 (prophylactic positive group, four pens) were offered the grower feed containing 2 kg/tonne of the product (62 ppm) from 4 days before they were challenged with *B. hyodysenteriae* and thereafter throughout the experiment. Pigs in each of groups 2 and 3 were challenged with *B. hyodysenteriae* and received medicated feed (treatment) from the day the first pig in a particular pen developed diarrhoea (faecal score 3): pigs in group 2 received 2 kg/tonne of the product and those in group 3 received 4 kg/tonne (124 ppm). Once medication was started for pens of pigs in groups 2 and 3, this was continued until the end of the experiment. Group 4 consisted of four pens of pigs that were challenged with *B. hyodysenteriae* but did not receive medicated feed (positive control group). Pigs in group 5 (two pens) received no medication in the diet and were not challenged with *B. hyodysenteriae* (negative control group). Pigs in group 6 (two pens) received the weaner diet with 2 kg/tonne of the product added and were not challenged with *B. hyodysenteriae* (product negative control).

For groups 1–4, half of the subgroups were contained in room A and half in room B (24 pigs in each room). The four pens for the 12 pigs in groups 5 and 6 (no experimental challenge) were all contained in room C. Strict biosecurity protocols, including the use of different sets of protective clothing in the different rooms, were maintained to prevent possible transmission of infection between the rooms and pens.

Experimental infection. Experimental infection for all pigs that were to be challenged (groups 1–4) commenced 4 days after the pigs in groups 1 and 6 started to receive the prophylactically medicated grower diet. Australian *B. hyodysenteriae* isolate '13' was recovered from frozen storage and propagated on TSA and in Kunkle's prereduced anaerobic broth.[14] Experimental infection was by oral gavage. Each pig receiving a slurry containing 100 mL of mid-log phase broth culture and four diced TSA plates on which the spirochaete was growing mixed into the broth, to give approximately 10^{10} spirochaete cells/pig. This challenge was repeated on the next 2 days.

Table 3. Minimum inhibitory concentrations (MICs) of three antimicrobials for 31 recent Australian isolates of *Brachyspira hyodysenteriae* and reference strain WA1, and single nucleotide polymorphisms in the sequence of their 23S rRNA genes at positions equivalent to 2058 and 2059 in *Escherichia coli*

Isolate	MIC (μg/mL)			Mutations	
	Kitasamycin	Tylosin	Lincomycin	A2058	A2059
1	≥40 < 80	>1 < 4	<2	—	—
2	≥40 < 80	≥50 < 100	≥72	T	—
3	≥40 < 80	≥100	≥72	G	—
4	≥40 < 80	≥100	≥72	T	—
5	≥40 < 80	≥100	≥16 < 36	—	—
6	≥10 < 20	≥100	<2	T	—
7	≥10 < 20	≥100	≥72	NA[a]	NA
8	≥10 < 20	≥100	≥36 < 72	T	—
9	≥10 < 20	≥100	≥36 < 72	T	—
10	≥1.25 < 2.5	≥100	<2	—	G
11	≥20 < 40	≥100	≥72	—	—
12	≥20 < 40	≥100	≥72	NA	NA
13[b]	≥2.5 < 5	≥1 < 4	<2	—	—
14	≥40 < 80	≥100	≥72	—	—
15	>5 ≤ 10	>4 ≤ 25	<2	—	G
16	≥20 < 40	≥100	≥72	—	—
17	≥20 < 40	≥100	≥36 < 72	NA	NA
18	≥40 < 80	≥50 < 100	≥72	T	—
19	≥40 < 80	≥100	≥72	T	—
20	≥20 < 40	≥100	≥72	T	—
21	>10 ≤ 20	>100	>4 ≤ 16	T	—
22	>160	>100	>72	T	—
23	>40 ≤ 80	>100	>72	T	—
24	>10 ≤ 20	>100	>36 ≤ 72	—	G
25	>2.5 ≤ 5	>1 ≤ 4	≤2	T	—
26	>2.5 ≤ 5	>1 ≤ 4	≤2	—	—
27	>160	>50 ≤ 100	>72	T	—
28	>20 ≤ 40	>100	>72	T	—
29	>160	>25 ≤ 50	>36 ≤ 72	—	G
30	>5 ≤ 10	>100	≤2	—	—
31	>10 ≤ 20	>100	>4 ≤ 16	T	—
WA1	≥20 < 40	>100	>72	T	—

[a]NA, sequence data not available. [b]Challenge strain used in the study.

Monitoring for clinical signs and faecal excretion of B. hyodysenteriae. Pigs were monitored for signs of disease on a daily basis. The consistency of the faeces as they were passed was scored as shown in Table 2. Faecal samples were collected from each pig prior to experimental infection and twice per week (every 3 or 4 days) commencing after experimental challenge. The samples were subjected to

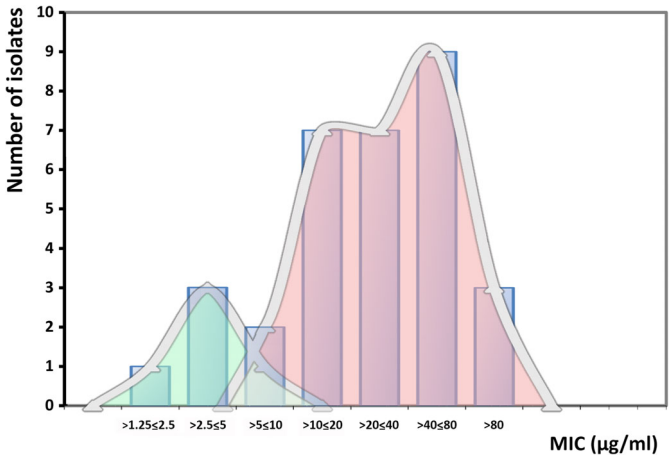

Figure 1. Minimum inhibitory concentration (MIC) distribution for kitasamycin among 31 Australian isolates of *Brachyspira hyodysenteriae* and strain WA1. Small curve on left denotes susceptible (wild type) isolates; large curve on right denotes resistant isolates; overlapping of curves denotes isolates with intermediate susceptibility.

anaerobic culture for *B. hyodysenteriae* using selective TSA containing 400 µg/mL of spectinomycin and 25 µg/mL each of colistin and vancomycin (Sigma–Aldrich, St. Louis, MO, USA).[15] Zones of strong haemolysis around the inoculated area indicated growth of *B. hyodysenteriae*, and confirmation was obtained that spirochaetes were present by resuspending surface growth in PBS and viewing with a phase-contrast microscope. The relative numbers of *B. hyodysenteriae* on the plates were scored 0–5, with 0 being no growth, 1 being strongly haemolytic growth in the first streak and so on until 5 which indicated strongly haemolytic growth out to the last (fifth) streak.

Pigs with mucohaemorrhagic colitis (faecal score 4) were removed for postmortem (pm) examination. On the day that the first pig of the unmedicated control pigs (group 4) in rooms A and B were killed because they had a faecal score of 4, a single pig in group 1 (receiving prophylactic medication) in the same room also was randomly selected and killed on the same day. All remaining pigs that did not develop disease were removed 5 weeks after the first day of the experimental challenge, apart from pig 41 in group 2 that was killed 28 days after challenge, because it developed a severe rectal prolapse. The pigs were stunned using a captive bolt pistol and exsanguinated. The intestinal tract was removed and the large intestine opened and macroscopically evaluated. Contents from the caecum and proximal colon were collected and subjected to selective anaerobic culture as described previously. Tissue sections from the proximal colon were excised and fixed in 10% neutral buffered formalin before being processed for histological examination. Paraffin embedded sections were cut at 5 µm and stained with haematoxylin and eosin. Sections were examined by an independent veterinary pathologist who was not informed of the treatment groups.

Analysis

For the animal trial, the pig was used as the unit of statistical analysis. Fisher's exact test was used to make comparisons between groups in the numbers of pigs developing SD (score 4) or not. Median values were calculated for each pig and group for faecal condition scores and faecal excretion of *B. hyodysenteriae* which were measured twice per week. Group medians were calculated for the presence of *B. hyodysenteriae* in the faeces, caecum and colon at pm. The results for unchallenged groups 5 and 6 were combined, as they did not differ (all values 0). The distributions of the overall group medians for the measured parameters were compared using the Kruskal–Wallis test, and where significant differences were observed the Mann–Whitney U test (two tailed) was used for pairwise comparisons of groups to determine which were significantly different from each other.

Results

Antimicrobial susceptibility

The MICs for kitasamycin, tylosin and lincomycin for the 31 Australian isolates of *B. hyodysenteriae* and strain WA1 are shown in Table 3. Using the ECOFFs established by Pringle et al. for *B. hyodysenteriae*,[16] only four isolates were susceptible to tylosin (<16 µg/mL), with another one possibly susceptible (>4 < 25 µg/mL), and eight were susceptible to lincomycin (<2 µg/mL). There are no clinical breakpoints or epidemiological (wild type) cut-off values available for kitasamycin. The distribution of the 31 isolates and WA1 with respect to their MIC to kitasamycin is shown in Figure 1. The ECOFF was estimated as ≤5 µg/mL, with four isolates (12.9%) considered to be susceptible (wild type). Unexpectedly, one of these susceptible isolates, which was also susceptible to tylosin, had an A2058T transversion (Tables 3 and 4). Two isolates (6.5%) with

Table 4. Distribution of susceptibility to kitasamycin and tylosin and presence of single nucleotide polymorphisms at positions 2058 and 2059 on the 23S rRNA gene of the 31 *Brachyspira hyodysenteriae* isolates[a]

Kitasamycin[b]	Tylosin[c]	T2058	G2058	G2059	NA[d]	Number of isolates
R	R	+	–	–	–	14
R	R	–	+	–	–	1
R	R	–	–	+	–	2
R	R	–	–	–	–	4
R	R	–	–	–	+	3
R	S	–	–	–	–	1
I	R	–	–	–	–	1
S	R	–	–	+	–	1
I	I	–	–	+	–	1
S	S	+	–	–	–	1
S	S	–	–	–	–	2

[a]Strain WA1 also was resistant to both macrolides and had the A2058T transversion. [b]Susceptibility to kitasamycin according to epidemiological cut-off values (ECOFFs) derived from Figure 1. R, resistant (>10 µg/mL); I, intermediate (>5 ≤ 10 µg/mL); S, sensitive (≤5 µg/mL). [c]Susceptibility to tylosin using the ECOFFs of Pringle et al.[16] R, resistant (>16 µg/mL); I, intermediate (>4 < 16 µg/mL: this study); S, susceptible (≤4 µg/mL). [d]NA, sequence data not available for these three isolates.

Table 5. Detection of abnormal faeces in pigs in the six experimental groups[a]

Pig number	Regimen and group	Room	Days postchallenge													
			7	10	13	14	15	17	18	19	21	22	24	28	31	Median
1	Prophylactic 62 ppm (1)	A	0	0	0	0	0	1	1	1	1	1	1	1	1	1
2	Prophylactic 62 ppm (1)	A	0	0	0	1	1	1	1	1	1	1	0	0	1	1
3	Prophylactic 62 ppm (1)	A	0	1	1	Dead										1
13	Prophylactic 62 ppm (1)	A	0	0	1	1	1	1	1	1	1	1	1	1	1	1
14	Prophylactic 62 ppm (1)	A	0	0	0	0	1	1	1	1	0	0	0	1	0	0
15	Prophylactic 62 ppm (1)	A	0	0	0	0	0	0	0	1	2	2	2	1	1	1
34	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	0	0	1	1	1	1	1	0
35	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	0	0	0	0	0	1	0	0
36	Prophylactic 62 ppm (1)	B	0	0	1	Dead										0
46	Prophylactic 62 ppm (1)	B	0	0	1	1	1	1	1	1	1	1	1	1	1	1
47	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	1	1	1	1	1	1	1	1
48	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Median			0	0	0	0	0	0.5	1	1	1	1	1	1	1	1[b]
4	Therapeutic 62 ppm (2)	A	0	0	0	1	1	2	2	**3+**[c]	3	2	1	1	1	1
5	Therapeutic 62 ppm (2)	A	0	0	0	1	1	2	2	**2+**	2	2	1	1	1	1
6	Therapeutic 62 ppm (2)	A	0	0	1	1	1	1	1	**1+**	2	1	1	0	0	1
22	Therapeutic 62 ppm (2)	A	0	1	1	1	**3+**	1	1	1	2	2	2	1	1	1
23	Therapeutic 62 ppm (2)	A	0	0	0	1	**1+**	1	1	1	1	1	1	1	1	1
24	Therapeutic 62 ppm (2)	A	0	0	0	0	**0+**	0	0	0	0	0	1	1	1	0
25	Therapeutic 62 ppm (2)	B	0	0	0	0	1	2	**2+**	2	2	1	1	0	0	1
26	Therapeutic 62 ppm (2)	B	0	0	0	0	1	1	**1+**	2	1	1	1	1	0	1
27	Therapeutic 62 ppm (2)	B	0	0	0	1	1	1	**3+**	3	2	2	2	2	2	1.5
43	Therapeutic 62 ppm (2)	B	0	1	2	**3+**	3	3	3	2	2	2	2	1	1	2
44	Therapeutic 62 ppm (2)	B	0	1	2	**2+**	2	2	2	2	1	1	0	1	0	1
45	Therapeutic 62 ppm (2)	B	0	0	2	**2+**	2	2	2	2	0	1	0	0	0	1
Median			0	0	0	1	1	1.5	2	2	2	1	1	1	1	1[b]
7	Therapeutic 124 ppm (3)	A	0	1	**3+**	3	3	2	2	1	1	1	1	1	1	1
8	Therapeutic 124 ppm (3)	A	0	0	**2+**	1	1	1	1	1	1	1	1	1	1	1
9	Therapeutic 124 ppm (3)	A	0	0	**0+**	0	0	0	0	0	1	1	1	1	1	0
16	Therapeutic 124 ppm (3)	A	0	0	0	1	1	**0+**	1	1	1	1	1	1	0	1
17	Therapeutic 124 ppm (3)	A	0	0	1	1	2	**0+**	2	1	2	2	1	1	1	1
18	Therapeutic 124 ppm (3)	A	0	0	1	2	2	**3+**	3	3	2	2	1	1	1	2
31	Therapeutic 124 ppm (3)	B	0	0	0	0	0	0	1	1	2	**3+**	2	1	1	1
32	Therapeutic 124 ppm (3)	B	0	0	0	0	0	0	0	0	0	**0+**	1	1	1	0
33	Therapeutic 124 ppm (3)	B	0	0	0	0	0	0	0	0	0	**0+**	0	1	1	0
40	Therapeutic 124 ppm (3)	B	0	0	0	**0+**	0	0	0	0	0	0	0	0	0	0
41	Therapeutic 124 ppm (3)	B	0	0	2	**2+**	1	1	1	1	2	2	1	Dead		1
42	Therapeutic 124 ppm (3)	B	0	1	2	**3+**	1	1	1	1	2	2	2	1	1	1
Median			0	0	1	**1**	1	0	1	1	1	1	1	1	1	1[b]
10	+ve control (4)	A	2	2	2	2	2	2	2	2	3	3	3	Dead		2
11	+ve control (4)	A	1	3	4	Dead										2
12	+ve control (4)	A	2	2	2	3	3	3	3	3	3	3	3	Dead		3
19	+ve control (4)	A	0	2	3	3	3	3	3	3	3	4	Dead			3
20	+ve control (4)	A	0	0	0	0	0	0	2	2	3	3	3	2	2	2
21	+ve control (4)	A	2	2	3	3	3	3	3	3	3	3	Dead			3
28	+ve control (4)	B	0	0	3	3	3	3	3	3	3	3	3	Dead		3
29	+ve control (4)	B	3	4	4	Dead										3.5

Table 5. Continued

Pig number	Regimen and group	Room	Days postchallenge												
			7	10	13	14	15	17	18	19	21	22	24	28	31	Median
30	+ve control (4)	B	3	3	3	3	3	3	3	3	3	3	Dead			3
37	+ve control (4)	B	2	3	3	3	3	3	3	3	4	4	Dead			3
38	+ve control (4)	B	2	2	3	3	3	3	3	3	4	4	Dead			3
39	+ve control (4)	B	0	0	0	0	0	0	0	0	0	0	0	2	1	0
Median			2	2	3	3	3	3	3	3	3	3	3	3	1.5	3[b]
49	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
51	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
56	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Median			0	0	0	0	0	0	0	0	0	0	0	0	0	0[b]
52	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
54	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
58	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
59	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Median			0	0	0	0	0	0	0	0	0	0	0	0	0	0[b]

[a]The faecal scoring system (0–4) is shown in Table 2. The faeces were observed daily, but observations are only shown and analysed for days when faeces were cultured (Table 7), and on the days where medication was started. Pigs 11 and 29 in the positive control group were killed when faecal score 4 occurred. Pig 41 was killed because of a rectal prolapse. Two pigs in the prophylactic group (pigs 3 and 36) were killed on the same day that the first pig in the positive control group in the same room was killed. [b]In each case, the median of the medians relates to the scores for the group presented in the column above this figure. The results of all the statistical comparisons are summarized in the text of the results in the main manuscript. [c]Crosses in bold cells indicate the first day that medication was started in that medicated group of three pigs because at least one pig had a faecal score of 3 (diarrhoea).

MICs of >5 and ≤10 μg/mL were scored as 'intermediate', because they were located in the overlapping region between the two MIC curves (one had an A2059G transversion). It was not clear whether they should be considered above or below the ECOFF. The other 25 isolates (80.6%) were considered to be resistant to kitasamycin based on the MIC values, although not all showed corresponding mutations in the 23S rRNA gene (Tables 3 and 4).

Twenty-four isolates (77.4%) were resistant to both kitasamycin and tylosin, and another isolate was in the intermediate category with respect to both these macrolides (Table 4). One isolate that was resistant to tylosin was in the intermediate zone for kitasamycin, and another tylosin resistant isolate was sensitive to kitasamycin. One isolate was resistant to kitasamycin but not to tylosin. Only three isolates (9.7%) were susceptible to both macrolides.

23S rRNA gene sequences

SNPs were found in the 23S rRNA gene at positions A2058 and A2059 in 20 of the 28 isolates (71.4%) for which sequence data were available (Tables 3 and 4). A2058T transversions were found in 15 isolates, of which 14 were resistant to both macrolides and one isolate was sensitive to both macrolides. An A2058G transversion was found in a single isolate and this was resistant to both macrolides. An A2059G transversion was found in four isolates, of which two were resistant to both macrolides, one was recorded as intermediate for both macrolides and one was susceptible to kitasamycin but resistant to tylosin. Strain WA1 had an A2058T transversion and was resistant to both macrolides (and to lincomycin). Over all the isolates, these SNPs were aligned but not perfectly correlated with reduced susceptibility to either of the macrolides or to lincomycin. Other SNPs were found in the 23S rRNA genes (289 SNIPS for the 28 isolates, and three for WA1), but none were obviously correlated with resistance (Table S1, Supporting information).

Animal trial

Commencement of medication due to disease. The day on which medication was commenced for the pigs in groups 2 and 3 is shown in Table 5. This varied from day 13 to day 22 after the first day of experimental challenge.

Clinical signs. Ten of the 12 pigs that were challenged with *B. hyodysenteriae* but did not receive medicated food (group 4: the positive control group) developed mucohaemorrhagic diarrhoea (SD) and were removed for pm (Table 5). These comprised 5/6 pigs in pens from each of rooms A and B. None of the pigs in the other

Table 6. ***Brachyspira hyodysenteriae*** **culture results from faeces in the period following challenge and from the faeces, caecum and colon at postmortem examination**[a]

Pig number	Regimen and group	Room	Days postchallenge								Median	Postmortem samples		
			7	10	14	17	21	24	28	31		Faeces	Caecum	Colon
1	Prophylactic 62 ppm (1)	A	0	0	0	1	1	1	1	1	1	0	0	0
2	Prophylactic 62 ppm (1)	A	0	0	1	1	1	0	0	1	0.5	0	0	1
3	Prophylactic 62 ppm (1)	A	0	1	Dead[b]						1	0	0	0
13	Prophylactic 62 ppm (1)	A	0	0	1	1	1	1	1	1	1	1	0	1
14	Prophylactic 62 ppm (1)	A	0	0	0	1	0	0	1	0	0	0	0	0
15	Prophylactic 62 ppm (1)	A	0	0	0	0	2	2	1	1	0.5	1	1	1
34	Prophylactic 62 ppm (1)	B	0	0	0	0	1	1	1	1	0.5	1	0	1
35	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	1	0	0	0	0	1
36	Prophylactic 62 ppm (1)	B	0	0	Dead[b]						0	0	0	0
46	Prophylactic 62 ppm (1)	B	0	0	1	1	0	0	0	0	0	0	1	1
47	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	0	0	0	0	0	0
48	Prophylactic 62 ppm (1)	B	0	0	0	0	0	0	0	0	0	0	0	0
Median			0	0	0	0.5	0.5	0	1	0.5	0.5[c]	0	0	0.5
4	Therapeutic 64 ppm (2)	A	0	0	3	5	**3**[d]	1	1	1	1	1	1	1
5	Therapeutic 64 ppm (2)	A	0	0	1	5	**5**	3	1	1	1	0	0	0
6	Therapeutic 64 ppm (2)	A	0	0	1	1	**1**	1	0	0	0.5	0	0	0
22	Therapeutic 64 ppm (2)	A	0	5	4	**4**	2	2	1	1	2	1	1	1
23	Therapeutic 64 ppm (2)	A	0	0	4	**4**	1	1	1	1	1	0	1	0
24	Therapeutic 64 ppm (2)	A	0	0	0	**0**	0	1	1	1	0	0	0	0
25	Therapeutic 62 ppm (2)	B	0	0	0	5	**5**	3	2	0	1	0	0	1
26	Therapeutic 62 ppm (2)	B	0	0	0	4	**3**	1	1	0	0.5	0	0	1
27	Therapeutic 62 ppm (2)	B	0	0	1	1	**2**	2	2	2	1.5	1	1	1
43	Therapeutic 62 ppm (2)	B	5	5	5	**3**	2	2	2	1	2	1	0	1
44	Therapeutic 62 ppm (2)	B	0	0	0	**0**	0	0	0	0	0	0	0	0
45	Therapeutic 62 ppm (2)	B	0	0	0	**0**	0	0	0	0	0	0	0	0
Median			0	0	1	3.5	2	1	1	1	1[c]	0	0	0.5
7	Therapeutic 124 ppm (3)	A	0	**3**	5	2	1	1	1	1	1	0	0	0
8	Therapeutic 124 ppm (3)	A	0	**0**	3	3	1	1	1	1	1	1	0	1
9	Therapeutic 124 ppm (3)	A	0	**0**	0	0	1	0	1	1	0	0	0	0
16	Therapeutic 124 ppm (3)	A	0	0	0	**3**	1	1	1	0	0.5	0	0	0
17	Therapeutic 124 ppm (3)	A	0	0	4	**5**	2	1	1	1	1	0	0	0
18	Therapeutic 124 ppm (3)	A	0	0	5	**5**	4	2	2	2	2	1	1	1
31	Therapeutic 124 ppm (3)	B	0	0	0	0	5	**5**	4	3	1.5	1	1	1
32	Therapeutic 124 ppm (3)	B	0	0	0	0	0	**1**	1	1	0	0	0	0
33	Therapeutic 124 ppm (3)	B	0	0	0	0	0	**0**	1	1	0	0	0	0
40	Therapeutic 124 ppm (3)	B	0	0	0	**0**	0	0	0	0	0	0	0	0
41	Therapeutic 124 ppm (3)	B	5	5	5	**3**	2	1	Dead		4	1	1	1
42	Therapeutic 124 ppm (3)	B	3	5	4	**3**	2	1	1	1	2.5	1	0	1
Median			0	0	1.5	2	1	1	1	1	1[c]	0	0	0
10	+ve control (4)	A	5	5	5	5	5	5	Dead		5	4	3	5
11	+ve control (4)	A	5	5	Dead						5	4	5	5
12	+ve control (4)	A	0	0	5	5	5	5	Dead		5	4	5	5
19	+ve control (4)	A	0	5	5	5	5	Dead			5	4	3	5
20	+ve control (4)	A	0	0	0	0	3	3	4	2	1	1	5	5
21	+ve control (4)	A	5	5	5	5	5	Dead			5	4	4	5
28	+ve control (4)	B	0	0	5	5	5	5	Dead		5	4	5	5

Table 6. Continued

Pig number	Regimen and group	Room	Days postchallenge								Median	Postmortem samples		
			7	10	14	17	21	24	28	31		Faeces	Caecum	Colon
29	+ve control (4)	B	5	5	Dead						5	4	5	5
30	+ve control (4)	B	3	5	5	5	5	Dead			5	4	5	5
37	+ve control (4)	B	0	5	5	5	5	Dead			5	4	5	5
38	+ve control (4)	B	0	5	5	5	5	Dead			5	4	5	5
39	+ve control (4)	B	0	0	0	0	0	0	2	1	0	1	4	5
Median			0	5	5	5	5	5	3	1.5	5[c]	4	5	5
49	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
50	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
51	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
55	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
56	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
57	−ve control (5)	C	0	0	0	0	0	0	0	0	0	0	0	0
Median			0	0	0	0	0	0	0	0	0[c]	0	0	0
52	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
53	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
54	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
58	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
59	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
60	Prophylactic 62 ppm (6)	C	0	0	0	0	0	0	0	0	0	0	0	0
Median			0	0	0	0	0	0	0	0	0[c]	0	0	0

[a]Scores 0 to 5, depending on the number of streaks positive for strongly haemolytic growth on the isolation plate. All plates scored zero at the preinfection sampling. [b]Healthy pig in the infected prophylactic group killed at the same time as the first pig in the positive control group in that room was removed, because it had developed mucohaemorrhagic colitis. Pig 41 was killed, because it had a severe rectal prolapse. [c]In each case, the median of the medians relates to the scores for the group presented in the column above this figure. The results of all the statistical comparisons are summarised in the text of the results in the main manuscript. [d]Sets of three bold cells in a column indicate the first sampling day after medication had started for that pen of pigs. For actual day of the start of medication for these pigs, see Table 5.

five groups developed clinical SD or had pm signs consistent with SD, and for each group, the difference compared with unmedicated group 4 was highly significant (P = 0.0001: Fisher's exact test).

None of the challenged pigs that received the prophylactically medicated feed (group 1) developed diarrhoea; however, two were killed as controls for the first unmedicated pigs with SD in the same room. At least one of the pigs per pens in the eight therapeutic subgroups (groups 2 and 3) showed diarrhoea that triggered the whole pen being offered medicated feed. Subsequently, none of these animals went on to develop mucohaemorrhagic diarrhoea, and the diarrhoea that was present abated. The longest time after medication started that faeces with score 3 were still recorded was 4 days (pig 43 in group 2, receiving 2 kg/tonne).

Comparison of the distribution of clinical scores between the groups indicated a significant group effect when the medians of the medians were compared (P < 0.01; Kruskal–Wallis test), and pairwise comparisons of group medians in the Mann–Whitney test showed that clinical scores for all the four infected groups (1–4) were significantly higher than the scores for the unchallenged groups 5 and 6 (P < 0.01), except for group 1 where the significance was P < 0.05). Differences between medicated groups 1, 2 and 3 were not significant, but the distribution of clinical scores for group 4 (unmedicated) was significantly greater than that for all the other groups (all P < 0.01).

Faecal excretion and postmortem occurrence of B. hyodysenteriae. The pattern of faecal excretion of *B. hyodysenteriae* in individual pigs with time, and its occurrence in pm samples (faeces, caecum and colon) in the six groups are shown in Table 6.

As expected, none of the unchallenged pigs in room C (groups 5 and 6) shed *B. hyodysenteriae* in their faeces. All but two of the pigs receiving kitasamycin prophylactically (group 1) shed low numbers of *B. hyodysenteriae* on at least 1 day, and some pigs shed low numbers for up to 2 weeks (e.g. pigs 1, 13, 15 and 34). Following kitasamycin medication at either 62 or 124 ppm (groups 2 and 3, respectively), the numbers of *B. hyodysenteriae* in the faeces of the pigs declined, but only 2/12 and 1/12 in the two groups, respectively, did not show any faecal excretion over the experimental period.

Heavy growths of *B. hyodysenteriae* were isolated from the caecum and colon of the 10 unmedicated positive control pigs of group 4 that had mucohaemorrhagic colitis, as well as from positive control pig 39 which had moderate microscopic changes in the colon consistent

Table 7. Gross and histological findings in the caecum and colon of the experimental pigs[a]

Pig number	Killed due to disease[b]	Regimen and group	Gross pathology (caecum and colon)	Colon	Histopathology (colon)		Increased neutrophils in lamina propria	Inflammation	Other[c]
			Caecum		Increased mucosal thickness (>50%)	Surface epithelial injury			
1	–	Prophylactic (1)	Normal	Normal	——	——	——	——	——
2	–	Prophylactic (1)	Normal	Normal	—	Rare	Mild	Mild	—
3	–	Prophylactic (1)	Normal	Normal	—	Rare	—	—	—
13	–	Prophylactic (1)	Normal	Slight erythema	—	—	—	—	—
14	–	Prophylactic (1)	Normal	Normal	—	—	—	—	—
15	–	Prophylactic (1)	Slight erythema	Slight erythema in mid colon	—	—	—	—	—
34	–	Prophylactic (1)	Mild erythema top half	Mild erythema middle half	—	—	—	—	—
35	–	Prophylactic (1)	Normal	Patchy erythema	—	Rare	Mild	Mild	—
36	–	Prophylactic (1)	Normal	Slight erythema	—	Rare	Mild	Mild	—
46	–	Prophylactic (1)	Mild/moderate erythema	Mild erythema	—	—	—	—	—
47	–	Prophylactic (1)	Normal	Normal	—	—	—	—	—
48	–	Prophylactic (1)	Slight erythema	Patchy erythema middle half	—	Rare	—	—	—
4	–	Therapeutic 62 ppm (2)	Slight erythema	Slight erythema, middle half	—	—	—	—	—
5	–	Therapeutic 62 ppm (2)	Slight erythema	Normal	—	—	—	—	—
6	–	Therapeutic 62 ppm (2)	Normal	Normal	—	—	—	—	—
22	–	Therapeutic 62 ppm (2)	Slight erythema	Slight erythema mid/lower colon	—	—	—	—	—
23	–	Therapeutic 62 ppm (2)	Slight erythema	Patchy erythema entire length	—	—	—	Mild	—
24	–	Therapeutic 62 ppm (2)	Slight erythema	Normal	—	—	—	—	—
25	–	Therapeutic 62 ppm (2)	Normal	Normal	—	—	—	—	—
26	–	Therapeutic 62 ppm (2)	Slight erythema	Patchy erythema entire length	—	—	—	—	—
27	–	Therapeutic 62 ppm (2)	Moderate erythema	Mild–moderate erythema entire length	—	—	—	—	—
43	–	Therapeutic 62 ppm (2)	Slight erythema	Mild erythema middle half	—	—	—	—	—
44	–	Therapeutic 62 ppm (2)	Normal	Normal	—	—	—	—	—
45	–	Therapeutic 62 ppm (2)	Normal	Normal	—	—	—	Mild (patchy)	—
7	–	Therapeutic 124 ppm (3)	Normal	Normal	—	—	Mild	Mild	—
8	–	Therapeutic 124 ppm (3)	Normal	Slight erythema mid-colon	—	—	—	—	—
9	–	Therapeutic 124 ppm (3)	Normal	Normal	—	—	Mild	Mild	—

Table 7. Continued

Pig number	Killed due to disease[b]	Regimen and group	Gross pathology (caecum and colon)	Colon	Histopathology (colon)		Increased neutrophils in lamina propria	Inflammation	Other[c]
			Caecum		Increased mucosal thickness (>50%)	Surface epithelial injury			
16	–	Therapeutic 124 ppm (3)	Normal	Normal	—	—	—	—	
17	–	Therapeutic 124 ppm (3)	Normal	Patchy erythema mid/lower colon	—	—	Mild	—	
18	–	Therapeutic 124 ppm (3)	Slight erythema	Patchy erythema mid/lower colon	—	—	Mild	Mild	
31	–	Therapeutic 124 ppm (3)	Mild erythema	Mild erythema	—	—	—	—	
32	–	Therapeutic 124 ppm (3)	Normal	Normal	—	Rare	—	—	
33	–	Therapeutic 124 ppm (3)	Normal	Normal	—	—	—	—	
40	–	Therapeutic 124 ppm (3)	Normal	Normal	—	Mild	—	Mild	—
41	–[d]	Therapeutic 124 ppm (3)	Slight erythema	Slight erythema middle half	—	Rare	—	—	—
42	–	Therapeutic 124 ppm (3)	Slight erythema top quarter	Slight erythema entire length	—	—	—	—	—
10	+	+ve control (4)	Normal	SMHC + M entire length	55%	Mild	Moderate	Mild/moderate	CB
11	+	+ve control (4)	SMHC + F + M	SMHC + F + M entire length	70%	Moderate	Moderate	Mild	CB
12	+	+ve control (4)	Normal	SMHC + M entire length	60%	Mild	Moderate	Mild	CB
19	+	+ve control (4)	Normal	SMHC + M entire length	60%	Mild	Marked	Moderate	CB
20	–	+ve control (4)	Patchy erythema	Normal	—	—	—	Mild	—
21	+	+ve control (4)	Normal	SMHC + M lower 3/4 colon	65%	Mild	Mild	Mild	CB
28	+	+ve control (4)	Normal	SMHC + M entire length	60%	Moderate	Moderate	Mild/moderate	CB
29	+	+ve control (4)	Moderate	SMHC + F + M entire length	65%	Moderate	Moderate	Mild/moderate	CB
30	+	+ve control (4)	Normal	Moderate to SMHC + M entire length	65%	Moderate	Moderate	Mild/moderate	CB
37	+	+ve control (4)	Normal	SMHC + M entire length	70%	Moderate	Moderate	Moderate	CB
38	+	+ve control (4)	Normal	SMHC + M entire length	65%	Moderate	Severe	Moderate	CB
39	–	+ve control (4)	Patchy erythema	Normal	55%	Moderate	Moderate	Moderate	CB
49	–	–ve control (5)	Normal	Normal	—	—	—	Mild	—
50	–	–ve control (5)	Normal	Normal	—	—	—	—	—
51	–	–ve control (5)	Normal	Normal	—	—	—	—	—
55	–	–ve control (5)	Normal	Normal	—	—	Mild (patchy)	Mild	—
56	–	–ve control (5)	Normal	Normal	—	—	Mild (patchy)	Mild (patchy)	—
57	–	–ve control (5)	Slight erythema	Normal	—	—	—	—	—
52	–		Patchy erythema	Patchy erythema	—	—	—	—	—

Table 7. Continued

Pig number	Killed due to disease[b]	Regimen and group	Gross pathology (caecum and colon)	Colon	Histopathology (colon)		Increased neutrophils in lamina propria	Inflammation	Other[c]
			Caecum		Increased mucosal thickness (>50%)	Surface epithelial injury			
		Prophylactic 62 ppm (6)							
53	–	Prophylactic 62 ppm (6)	Patchy erythema	Patchy erythema	—	—	—	—	—
54	–	Prophylactic 62 ppm(6)	Normal	Normal	—	Rare	—	Mild	—
58	–	Prophylactic 62 ppm (6)	Normal	Patchy erythema	—	—	—	—	—
59	–	Prophylactic 62 ppm (6)	Patchy erythema	Normal	—	—	—	Mild	—
60	–	Prophylactic 62 ppm (6)	Patchy erythema	Patchy erythema	—	—	—	—	—

with SD. Eighteen of the other 38 apparently healthy pigs that were challenged, that had received medicated feed and that did not have colonic changes consistent with SD had low numbers of *B. hyodysenteriae* in the caecum and/or colon at postmortem examination.

Comparison of the distribution of group medians using the Kruskal–Wallis test shows that there were significant differences in distribution between groups for faecal excretion of *B. hyodysenteriae*, and for pm faeces, caecum and colon *B. hyodysenteriae* scores (all $P < 0.01$). Pairwise comparisons of group medians in the Mann–Whitney test showed that the distribution of *B. hyodysenteriae* scores in the unmedicated challenged group 4 was significantly ($P < 0.01$) higher than that for all the other experimental groups for all four site comparisons (i.e. for the twice weekly faecal scores, and for pm faeces, caecum and colon scores). No significant differences occurred between the three medicated groups (1, 2 and 3) in any of the four site comparisons. No significant differences were found between the three medicated groups and the unchallenged groups (5 and 6) in the faeces at pm, or in the caecum, but the three medicated groups showed significant differences in the faeces medians ($P < 0.05$; <0.01 and <0.01, respectively) compared with the unchallenged groups (5 and 6). In addition, significant differences ($P < 0.05$) were found for the colon results for groups 1 and 2 (but not group 3), compared with the unchallenged groups 5 and 6.

Pathological findings at postmortem. At pm, the 10 unmedicated challenged pigs in group 4 that had mucohaemorrhagic diarrhoea also had a gross mucohaemorrhagic colitis. Of these, only one pig (number 11) had gross lesions consistent with SD in the caecum. The other two pigs in this group had a normal colonic appearance, but one pig (number 39) had microscopic changes similar to the other pigs with SD (Table 7). Pig 39 had only shown low-level faecal excretion of *B. hyodysenteriae* and mild diarrhoea on the last two sampling days, and it might have been progressing towards developing clinical SD. The gross appearance of the colons of all the other medicated pigs was normal. Erythema of varying intensity was observed in the wall of the colon in 19/38 (50%) of the medicated pigs that did not develop SD and in 18/38 (47.4%) of the caecal samples. This rate of occurrence was comparable to that in the unchallenged pigs (groups 5 and 6), where erythema was observed in the colons of 4/12 (33.3%) and the caecums of 5/12 (62.5%). In histology, the pigs with gross signs of mucohaemorrhagic colitis at pm also showed microscopic changes consistent with SD: these included a marked increase in crypt basophilia and crypt density with increased numbers of mitotic figures extending up to 2/3 of the height of the mucosa; goblet cell hyperplasia; superficial mucosal epithelial necrosis and erosion; neutrophilic colitis with superficial mucosal neutrophil exocytosis; and low numbers of mixed bacteria within the lumen and superficial mucosal crypts. Most pigs without gross lesions or just showing some erythema did not exhibit histological changes, although mild changes in some parameters were recorded in a few such animals from all groups, including those that had not been challenged (Table 7).

Discussion

The overall aim of this work was to determine whether kitasamycin would be an effective means to help treat and control SD in Australian pig herds. The first step in this process was to determine whether recent Australian isolates of *B. hyodysenteriae* were susceptible to kitasamycin in vitro. As there are no published ECOFFs for kitasamycin and *B. hyodysenteriae*, the distribution of the MIC

results for 31 recent Australian isolates and strain WA1 was evaluated. The resultant figure (Figure 1) identified two overlapping curves, with the population of isolates in the smaller curve to the left (with low MIC values) being regarded as 'wild type' (susceptible) and those in the larger curve to the right being resistant to kitasamycin. Isolates falling into the overlap of the two curves were recorded as intermediate. A better definition of the ECOFF for kitasamycin could be obtained by including many more isolates in the analysis and by using more closely spaced dilutions around the putative cut-off. In the current analysis, only four isolates fell into the definition of susceptible (MIC <5 μg/mL), but one of these had a SNP in the 23S rRNA gene that has been associated with macrolide resistance. A true wild type isolate should not show evidence of potential acquired resistance mechanisms. Two isolates were recorded as intermediate (MIC value >5 and ≤10), and one of these also had a resistance-associated SNP. Although the proportion of isolates that were apparently susceptible to kitasamycin was low (12.9%), it was the same number as for tylosin (four isolates). Interestingly, one isolate that was susceptible to kitasamycin was resistant to tylosin, and one that was susceptible to tylosin was resistant to kitasamycin. Another isolate that was resistant to tylosin was intermediate for kitasamycin, and one isolate was intermediate for both macrolides. Only eight isolates were susceptible to lincomycin (25.8%), emphasising how widespread resistance to macrolides and lincosamides is among *B. hyodysenteriae* isolates in Australia, as in other countries where similar studies have been undertaken.[13] It is interesting that strain WA1, isolated in the 1980s, also was resistant to both the macrolides and lincomycin. Hence, the occurrence of multiple resistance in Australian *B. hyodysenteriae* isolates is not a new phenomenon.

The first recognised mechanism of resistance to the macrolide tylosin by *B. hyodysenteriae* isolates was associated with a mutation in nucleotide position homologous with position 2058 of the *E. coli* 23S rRNA gene, with A substituted for T (A2058T), a change that is believed to reduce binding of the macrolide.[17] In a later study, decreased susceptibility to tylosin and lincomycin in *B. hyodysenteriae* isolates was associated with either an A2058T transversion or less frequently an A2058G transition on the 23S rRNA gene.[18] In the current study, the majority (16/28, 57.1%) of the isolates that were resistant to both macrolides had SNPs at A2058 (13 A2058T, one A2058G) or A2059G (two), but interestingly four isolates (14.3%) that were resistant to both macrolides (isolates 5, 10, 12 and 14) did not present these SNPs (Table 4). Strain WA1 also had the A2058T transversion. SNPs at other positions occurred in nearly all the isolates, but none were obviously consistently correlated with this lack of susceptibility (Table S1). Only three isolates were sensitive to both macrolides, and unexpectedly one of these had the A2058T transversion. As differences in sensitivities to the two macrolides occurred in a few isolates, this suggests that the binding affinity for the two macrolides may differ, and/or that other nucleotide changes may influence relative binding, and/or that other mechanisms of resistance may be present in some isolates. Efflux pumps are used by some other bacterial species to remove kitasamycin and other macrolides,[19,20] but this mechanism is not known to occur in *B. hyodysenteriae*.

The experimental infection challenge that was used for the pigs was successful in inducing SD in most (10/12; 83%) of the challenged animals that did not receive a diet medicated with kitasamycin. On the other hand, a prophylactic rate of inclusion in the diet (2 kg/tonne), or therapeutic application of the same dose or of 4 kg/tonne given at the time that disease was starting, resulted in all pigs being protected from development of clinical SD. These differences in occurrence were highly significant (P = 0.0001) and provided good evidence that kitasamycin in the diet can be used to help control SD in the case of isolates that have a low MIC value to the drug. Comparison of the distribution of faecal condition scores between groups indicated that the four challenged groups (1–4) all had significantly higher scores than the unchallenged pigs, even though only pigs in group 4 developed SD. Hence, medication with kitasamycin in groups 1–3 did not completely prevent changes in faecal consistency in challenged pigs. Interestingly, no significant differences in faecal scores were found between the three medicated groups, indicating that prophylactic and therapeutic use of kitasamycin can result in similar levels of protection.

The disease control occurred despite the fact that many of the medicated pigs continued to excrete low numbers of *B. hyodysenteriae* in their faeces, and/or had low numbers of the spirochaete in their large intestinal contents at pm in the absence of disease or pathological changes. There were no significant differences between the three medicated groups in the presence of *B. hyodysenteriae* in their twice weekly faeces samples, or in the pm samples (faeces, caecum and colon samples). Hence, as with clinical signs, there was no clear comparative benefit of using kitasamycin either prophylactically or at two different therapeutic levels.

It is known that healthy pigs in some herds without disease may carry low numbers of virulent *B. hyodysenteriae* in their large intestines.[21,22] The lack of a complete bacteriological 'cure' at the dose rates of kitasamycin used in this experiment is a potential drawback, since these treated animals potentially still will be infectious. Furthermore, this experiment did not examine what would happen if kitasamycin therapy was withdrawn after successful treatment, and it cannot be excluded that disease would recur if the residual spirochaetes proliferated. Despite these caveats, the control achieved with oral kitasamycin should be useful in production herds with persistent SD problems that do not respond to other antimicrobials, but are demonstrated to be sensitive to kitasamycin. Veterinary diagnostic laboratories should be encouraged to include kitasamycin with other relevant antimicrobials when undertaking MIC testing on *B. hyodysenteriae* isolates. Of the three medication regimens tested, therapeutic application of oral kitasamycin at 62 ppm is recommended as it would limit total exposure to the macrolide while providing disease control.

Conclusions

The study confirmed the occurrence of widespread resistance to macrolides and lincosamides among Australian isolates of *B. hyodysenteriae*, including a strain from the 1980s. Four isolates with low MICs to the macrolide kitasamysin were identified among the 32 that were examined: three of these isolates also had low MICs to tylosin, whereas the fourth was resistant to tylosin. For most of the isolates, macrolide resistance was associated with mutations at

positions A2058 or A2059 on the 23S rRNA gene, although in some isolates, resistance was recorded in the absence of these changes. Additional mechanisms of resistance to these two macrolides may occur, and they may differ. When kitasamycin was incorporated into the feed at either 62 or 124 ppm and used either prophylactically or therapeutically, it was shown to prevent the occurrence of SD in pigs experimentally infected with an isolate of *B. hyodysenteriae* that had a low MIC to kitasamycin. Therapeutic application at 62 ppm was the preferred route of application, as it requires less antimicrobial use and was as effective as the other two options investigated. The increasing difficulties with controlling resistant strains of *B. hyodysenteriae* in Australia and elsewhere means that registration of kitasamycin for use as a therapeutic agent to control susceptible strains of *B. hyodysenteriae* should be considered.

Conflict of interest and sources of funding

The authors declare no conflicts of interest for the work presented here. HD is an employee of Apiam Animal Health, the company that funded the study.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.12876/suppinfo.

Table S1. Other mutations in the 23S rRNA genes for 28 *Brachyspira hyodysenteriae* isolates with sequence data, and for strain WA1.

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