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Chlorhexidine Susceptibility, Virulence Factors, and Antibiotic Resistance of Beta-Hemolytic *Escherichia coli* Isolated from Neonatal Swine with Diarrhea

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Since the discovery of penicillin in the late 1920's, hundreds of antimicrobial agents have been developed for human and animal use, dramatically reducing the morbidity and mortality associated with many infectious diseases. This use has resulted also in an unprecedented global increase in the incidence of bacterial pathogens that are resistant to multiple antibiotics. In addition to the use of antibiotics, strategies to control infection include the use of biocides in the form of antiseptics and disinfectants. Biocides are used widely in the food processing industry, human medicine, veterinary medicine, animal production and consumer's homes, and are composed of a variety of active ingredients in multiple combinations. There have been reports describing increased incidence of bacteria resistant to certain disinfectants recovered from environments where such agents have been used (Mølbak et al. 1999, Ferber 2000). Mechanisms of bacterial resistance to antibiotics have been widely studied; however, mechanisms of resistance to biocides are less well understood (Tattawasart et al. 1999). Resistance to biocides was considered to be mostly a result of native cellular mechanisms (intrinsic) rather than acquired resistance (White and McDermott 2001). Efflux mechanisms can be an important means of resistance for both antibiotics and biocides (Levy 2002). However, acquired resistance mechanisms are important, as plasmid-encoded genes have been shown to be responsible for biocide resistance (Brumfitt et al. 1985, Sidhu et al. 2002, Gilbert and McBain 2003). In a study of clinical isolates from Norway, acquired resistance played a larger role than expected, and was important in resistance to quaternary ammonium compounds (QACs) and co-resistance to antibiotics (Sidhu et al. 2002). Research reports have expressed concern that the use of biocides may be linked to the development of antibiotic resistance (Maris 1991, Sidhu et al. 2002). Although several laboratory studies have indicated such a link, the information is limited and the subject remains debatable. A link between antibiotic resistance and disinfectant resistance could potentially pose a greater challenge. Microorganisms, whether pathogenic or commensal, do find their way into runoff water, food processing and production, as well as in the clinical setting. We therefore sought to evaluate isolates obtained from sick animals with a biocide commonly used in human and veterinary medicine.

Chlorhexidine (1,1'-hexamethylenebis[5-(4-chlorophenyl)-biguanide]) is used widely in medical and veterinary applications as a topical antiseptic and disinfectant (Nicoletti et al. 1993). Antimicrobial activity of chlorhexidine and its mechanism of action have been reviewed (Beier et al. 2004). Chlorhexidine is used as a teat dip (Boddie et al. 1997), for veterinary wound management and skin preparation (Southwood and Baxter 1996), and in surgical scrub solutions (Southwood and Baxter 1996, Wan et al. 1997).

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Chlorhexidine is used in human medicine as a topical antiseptic, disinfectant, and in surgical scrub solutions (Foulkes 1973, Mulberry et al. 2001). Chlorhexidine recently has been tested for impregnating endotracheal tubes and urinary catheters (Chaiban et al. 2005).

The aim of this study was to (1) describe the distribution of chlorhexidine minimum inhibitory concentrations (MICs) for beta-hemolytic *E. coli* isolated from neonatal swine with diarrhea, (2) compare the MICs to ribogroup data, virulence factors, serotypes, and antibiotic resistance for the purpose of studying the relationship of antibiotic resistance and chlorhexidine resistance in these *E. coli*, and (3) determine whether these isolates contain linked antibiotic and disinfectant resistance.

MATERIALS AND METHODS

Eighty-nine beta-hemolytic *E. coli* were isolated from neonatal swine with diarrhea from multiple farms in Oklahoma over the time period from February 1998 – March 1999 (Bischoff et al. 2002). Bacterial isolates were stored as 20% glycerol stocks at –70°C until analyzed. Prior to performing broth microdilution testing on each isolate, glycerol stock cultures were streaked on Trypticase™ Soy Agar w/5% sheep blood (100 mm × 15 mm), BD BBL™ stacker™ plates (Becton, Dickinson and Company, Sparks, MD) and incubated at 37°C for 24 hr.

MICs were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) (formerly called NCCLS) guidelines of susceptibility tests for bacteria isolated from animals (CLSI 2002). The MICs were determined as the lowest concentration of compound that inhibited the visible growth of the microorganisms (Andrews 2001).

Chlorhexidine digluconate (2% chlorhexidine) was used for testing susceptibility to chlorhexidine. The method of susceptibility determination was similar to that used for triclosan susceptibility testing (Fan et al. 2002). Briefly, chlorhexidine was serially diluted twofold in Mueller Hinton broth (DIFCO brand Mueller Hinton Broth, No. 275730, Becton Dickinson and Company, Sparks, MD; Fisher Scientific, Houston, TX) across the wells of a 96-well plate through column 11 (Sterile 96-well microplates, U-shaped wells, Greiner, Longwood, FL). The chlorhexidine concentration ranged from 60 to 0.23 μg/mL. The wells in column 12 were used as positive control and contained only Mueller Hinton broth (50 μL). Following a 24-hr incubation, bacterial isolates were diluted with PBS-7 to a 0.5 McFarland Standard turbidity (Andrews 2001). The diluted bacteria (100 μL) were added to Mueller Hinton broth (9.9 mL) and 50 μL of the final bacterial solution was added to the diluted chemical and the control wells. The wells of the 96-well microplate were sealed with a sterile SealPlate™ adhesive sealing film (Excel Scientific, Wrightwood, CA), and the microplates were placed in a 37°C incubator for 24 hr. Following incubation, the results of susceptibility testing were obtained by visual inspection for bacterial growth in the wells with the aid of a reflective mirror Plate Reader (PGC Scientifics Corporation, Frederick, MD). These susceptibility tests were carried out in quadruplet and each determination was made on separate days to confirm consistency in susceptibility.

The *E. coli* isolates were assayed for susceptibility to 16 antimicrobials monitored by the National Antimicrobial Resistance Monitoring System (NARMS): amikacin, amoxicillin/clavulanic acid, ampicillin, ceftiofur, ceftriaxone, cephalothin,

chloramphenicol, ciprofloxacin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole. MICs were determined using the Sensititre automated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH, USA) and were interpreted according to CLSI breakpoints (CLSI 2002) unless the breakpoints were unavailable, in which case breakpoints in the NARMS 2002 Annual Report (NARMS 2002) were used. *E. coli* ATCC 25922 and ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms in antimicrobial MIC determinations, according to CLSI recommendations.

Ribotypes were obtained with the RiboPrinter Microbial Characterization System (Qualicon, Inc., Wilmington, DE) using the standard *Eco*RI DNA prep kit as described in the manufacturer's operations and analytical guides. Bacterial DNA was digested with *Eco*RI and gel electrophoresis was used to separate the restriction fragments into distinctive patterns. DNA patterns were hybridized with a chemiluminescent *E. coli* rRNA probe. The characterization system automatically placed patterns from each isolate into common ribogroups (clusters) based on their similarity to band position and intensity of patterns in the RiboPrinter's database. The ribogroups were analyzed and visually refined using the manufacturer's standard procedure.

Isolates were submitted to a commercial laboratory for O-antigen serotyping and virulence factor analysis (Gastroenteric Disease Center, Pennsylvania State University, Unversity Park, PA; http://ecoli.cas.psu.edu/). The *E. coli* isolates were analyzed for the following virulence factors: heat-labile toxin (LT), heat-stable toxin A (STa), heat-stable toxin B (STb), Shiga-like toxin I (STx1), Shiga-like toxin II (STx2), cytotoxic necrotizing factor 1 (CNF1), cytotoxic necrotizing factor 2 (CNF2), K88(F4) fimbriae (F4), K99(F5) fimbriae (F5), 987P fimbriae (F6), F107 fimbriae (F107), and the *E. coli* attaching-and-effacing factor (EAE).

Fisher's Exact Test was performed on data in Tables 1 and 2 using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA). The ordinal response of chlorhexidine MICs were analyzed using the Mantel-Haenszel Chi-Square Test to evaluate associations with antibiotic resistance using the SAS® system, version 8.02 (Stokes et al. 2000). A value of $P \leq 0.05$ was taken as significant.

RESULTS AND DISCUSSION

Observed chlorhexidine MICs for the *E. coli* isolates were 0.47 µg/mL for 50 of 89 strains, 0.94 µg/mL (5 strains), 1.88 µg/mL (23 strains), and 3.76 µg/mL (11 strains), which were two, four, and eightfold higher MICs, respectively, than the JM109 control strain and the other 50 isolated *E. coli* (Figure 1). Other researchers defined Staphylococci isolates as resistant if they were able to grow at or above a chlorhexidine level of 1 µg/mL (Leelaporn et al. 1994). Since we did not have a definition of resistance for *E. coli* to chlorhexidine we chose to refer to the isolates with higher chlorhexidine MIC levels as reduced-susceptible isolates. Thirty-nine of the *E. coli* isolates (43.8%) exhibited consistent reduced chlorhexidine susceptibility (MICs = 0.94, 1.88, and 3.76 µg/mL). The MIC distribution observed for our isolates was similar to the MIC distribution observed for hospital *E. coli* isolates, MICs = 0.5–8 µg/mL (Pitt et al. 1983) and 0.5–5 µg/mL (Hammond et al. 1987). However, the MIC distribution we observed was larger than the chlorhexidine MICs for a standard *E. coli* (MIC = 1 µg/mL) used by Wallhäusser (1984), or for *E. coli* isolated from broiler, cattle, and pig feces, MICs = 1–2 µg/mL (Aarestrup and Hasman 2004).

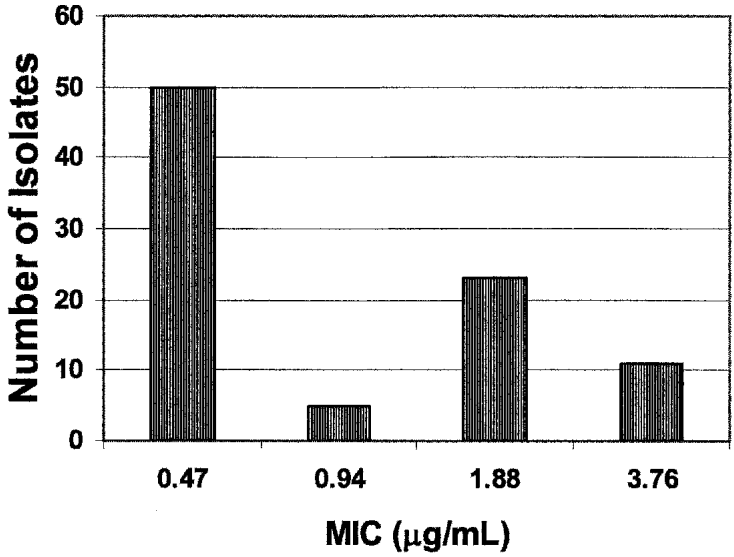

Figure 1. Distribution of chlorhexidine MICs among the swine *E. coli* isolates. The control *E. coli* strain, JM109 had an MIC = 0.47 μg/mL. Fifty isolates were susceptible to chlorhexidine at MIC = 0.47 μg/mL. Thirty-nine isolates showed reduced-susceptibility at MICs = 0.94 μg/mL (5), 1.88 μg/mL (23), and 3.76 μg/mL (11).

The 89 isolates were distributed over 19 different ribogroups. Seven ribogroups contained isolates with reduced chlorhexidine susceptibility, and most (84.6%) of the isolates primarily came from four ribogroups (126-S-5, 126-S-8, 128-S-6, and 130-S-8); each ribogroup contained a reduced-susceptible isolate ratio > 66% (Table 1). Ribogroup 126-S-5 contained 66.6% of the isolates that had reduced chlorhexidine susceptibility, and 86.7% of all isolates in ribogroup 126-S-5 expressed reduced chlorhexidine susceptibility (Table 1). The proportion of chlorhexidine susceptible and reduced-susceptible isolates in ribogroup 126-S-5 was considered significant compared to the proportion of isolates in ribogroup 127-S-5 using Fisher's Exact Test, P = 0.003 (Table 1). Also, the proportion of chlorhexidine susceptible and reduced-susceptible isolates in ribogroup 126-S-5 was considered significant when compared against the proportion of the sum of susceptible and sum of reduced-susceptible isolates in all the other six ribogroups combined using Fisher's Exact Test, P = 0.0009 (Table 1). In general, ribogroup data presents a fingerprint of the ribosomal DNA for the evaluated isolates. The results presented here suggest that there are molecular differences between isolates that are chlorhexidine susceptible and reduced-susceptible, and there is limited variability among the chlorhexidine reduced-susceptible isolates.

A high percentage of isolates with reduced chlorhexidine susceptibility were determined to have a combination of four virulence factor genes common to these isolates: STa, STb, STx2, and F107 (Table 2). It appeared that certain groups of virulence factors were consistently present in isolates with reduced chlorhexidine susceptibility (STa–STb–STx2; STa–STb–F107; STa–STb–STx2–F107; STb–STx2–F107; and STx2–F107) (Table 2). Twenty-seven of the 39 chlorhexidine reduced-

Table 1. Comparison of swine *E. coli* chlorhexidine susceptibility with ribogroup.

Ribogroup[a]	No. Susceptible Isolates [b]	No. Red.-Susc. Isolates [c]	% Red.-Susc. Isolates [d]	% Red.-Susc. of all Isolates [e]
126-S-1	6[h]	1[h]	2.6	14.3
126-S-5	4[f]	26[f]	66.6	86.7[g]
126-S-8	2[h]	4[h]	10.2	66.7[g]
127-S-5	7[h,i]	4[h,i]	10.2	36.4
127-S-6	1[h]	1[h]	2.6	50.0
128-S-6	0	2[h]	5.2	100.0[g]
130-S-8	0	1[h]	2.6	100.0[g]

[a] The chlorhexidine reduced-susceptible isolates (N = 39, MICs = 0.94 – 3.76 μg/mL) were found in 7 out of a total of 19 ribogroups.
[b] The Number of chlorhexidine susceptible isolates (MIC = 0.47 μg/mL) in these ribogroups.
[c] The number of chlorhexidine reduced-susceptible isolates in the ribogroups.
[d] % of Red.-Susc. Isolates = the percentage of chlorhexidine reduced-susceptible isolates in the ribogroup in comparison to the 39 total reduced-susceptible isolates.
[e] Percentage of chlorhexidine reduced-susceptible isolates in each ribogroup out of the total number of all isolates in the ribogroup.
[f] Using Fisher's Exact Test the two-sided P value was 0.003 between values with superscript *f* and superscript *i*.
[g] These 4 ribogroups contain 84.6% of the chlorhexidine reduced-susceptible isolates.
[h] Using Fisher's Exact Test the two-sided P value was 0.0009 between values with superscript *f* and the sum of values with superscript *h* in the corresponding columns.

susceptible isolates were in the indicated six pathotypes (shown with underlining) in Table 2, and each pathotype contained ≥ 75% reduced-susceptible isolates. Twenty-six of these 27 isolates had either serotype O147(15), or were not typeable, NT(11). The predominate pathotype that contained chlorhexidine reduced-susceptible isolates was STa–STb–STx2–F107 (serogroup O147), and the predominate pathotypes containing the chlorhexidine susceptible isolates were F107 (serogroups O138 and O139) and LT–STb–F4 (serogroups O8 and O149) (Table 2). The proportion of chlorhexidine susceptible and reduced-susceptible isolates in pathotypes F107 and LT–STb–F4 were significant compared to the proportion of isolates in pathotype STa–STb–STx2–F107 using Fisher's Exact Test, P < 0.0001 and < 0.0001, respectively (Table 2). Additionally, when the virulence factors STx2, F4, and F107 were found by themselves, they were primarily associated with the chlorhexidine susceptible isolates. Therefore, the chlorhexidine reduced-susceptible isolates did express a different set of virulence factors than were found in the susceptible isolates.

The chlorhexidine reduced-susceptible isolates demonstrated an increase in resistance to two antibiotics (Table 3). There is a significant association between the antibiotic resistance to gentamicin and streptomycin and with the chlorhexidine susceptibility, P = 0.04 and 0.04, respectively. The increased resistance to gentamicin and streptomycin by the chlorhexidine reduced-susceptible isolates demonstrates a potential link between antibiotic resistance and chlorhexidine susceptibility.

A link between the development of resistance to antiseptics and antibiotics was shown in animal-derived isolates by Maris (1991). Brumfitt et al. (1985) demonstrated that methicillin and gentamicin resistant *Staphylococcus aureus* (MGRSA) clinical isolates had increased resistance to chlorhexidine in comparison to non-resistant MGSA

Table 2. Occurrence of pathotypes and serotypes compared to chlorhexidine susceptibility in neonate swine *E. coli* isolates.

Pathotype[a]	No. Susc. Isolates [b]	No. Red.-Susc. Isolates [c]	% Red.-Susc. Isolates [d]	Serotype(s)[e]
STx2	1	0	0	
CNF1	5	3	37.5	O127(1), O11(1), O114(1)
F4	2	0	0	
F107	10[f,n]	2[g,n]	16.7	O147(1), O139(1)
EAE	1	0	0	
LT F4	1	0	0	
LT STb F4	22[h,i]	4[i,j]	15.4	O149(4)
LT STb F4 F6	1	0	0	
STa STb F4	1	0	0	
STa STb STx2[k]	0	1	100.0	NT(1)[l]
STa STb F107[k]	0	1	100.0	O147(1)
STa STb STx2 F107[k]	2[m,o]	18[m,o]	90.0	O147(11), NT(7)
STa STb STx2 F107[k] F5	0	1	100.0	NT(1)
STb F4	0	1	100.0	O98(1)
STb STx2 F107[k]	1	3	75.0	O147(2), NT(1)
STx2 F107[k]	1	3	75.0	O2(1), O147(1), NT(1)
No Factors	2	1	33.3	O35(1)
Not evaluated	0	1	100.0	

[a] Isolates were positive for these virulence factors: heat-labile toxin (LT), heat-stable toxin A (STa), heat-stable toxin B (STb), Shiga-like toxin II (STx2), cytotoxic necrotizing factor 1 (CNF1), K88(F4) fimbriae (F4), K99(F5) fimbriae (F5), 987P fimbriae (F6), F107 fimbriae (F107), and *E. coli* attaching-and-effacing factor (EAE).
[b] Chlorhexidine susceptible isolates (N = 50, MIC = 0.47 μg/mL).
[c] Chlorhexidine reduced-susceptible isolates (N = 39, MICs = 0.94 – 3.76 μg/mL).
[d] Percentage of chlorhexidine reduced-susceptible isolates of all isolates with the same virulence factor(s).
[e] The number of reduced-susceptible isolates of each serotype are listed in parentheses.
[f] These chlorhexidine susceptible isolates were in ribogroups 130-S-7(1), 126-S-1(6), 132-S-7(2) and 126-S-5(1), and had serogroups O138(1) and O139(7) or were NT(2)[l].
[g] These isolates are in ribogroups 126-S-1 (1) and 126-S-5 (1).
[h] These 22 chlorhexidine susceptible isolates had serogroups O8(3), O149(18) or were NT(1)[l], and were in the ribogroups 126-S-3(9), 127-S-5(5), and 127-S-8(8).
[i] Using Fisher's Exact Test the two-sided P value was < 0.0001 between values with superscripts *i* and *n*, and P is < 0.0001 between values with superscripts *i* and *o*.
[j] These isolates are in ribogroup 127-S-5.
[k] The underlining indicates groups of virulence factors that appear to be related to reduced-susceptibility.
[l] NT, not typeable.
[m] These isolates are in ribogroup 126-S-5.

isolates. A good correlation was found between chlorhexidine susceptibility and gentamicin resistance in clinical isolates of Gram-negative bacteria, and the authors concluded that routine antibiotic resistance testing may give clinicians the ability to predict chlorhexidine susceptibility (Kõljalg et al. 2002). It was demonstrated that a significant number of clinical Gram-negative bacterial isolates were not growth-inhibited by concentrations of chlorhexidine used for disinfection of wounds or instruments (Mengistu et al. 1999).

Table 3. Comparison of antibiotic resistance with respect to chlorhexidine susceptibility per antibiotic in neonate swine *E. coli* isolates.[a]

	Chlorhexidine Susceptibility				
Antibiotic[b]	0.47[c]	0.94[c]	1.88[c]	3.76[c] (μg/mL)	P values[d]
	(No. Antibiotic Resistant/and % Antibiotic Resistant)				
AMI	0/0	0/0	0/0	0/0	*e*
AUG	2/4%	0/0	2/9%	0/0	*e*
AMP	19/38%	4/80%	6/26%	2/18%	0.12
TIO	1/2%	0/0	0/0	0/0	*e*
AXO	0/0	0/0	0/0	0/0	*e*
CEP	6/12%	2/40%	3/13%	1/9%	0.76
CHL	25/50%	2/40%	12/52%	8/73%	0.20
CIP	0/0	0/0	0/0	0/0	*e*
FFN	31/62%	3/60%	15/65%	9/82%	0.24
GEN	4/8%	0/0	5/22%	3/27%	0.04
KAN	41/82%	5/100%	20/87%	10/91%	0.44
NAL	0/0	0/0	0/0	0/0	*e*
STR	37/74%	5/100%	20/87%	11/100%	0.04
SMX	43/86%	5/100%	21/91%	10/91%	0.57
TET	46/92%	5/100%	23/100%	11/100%	0.14
COT	19/38	0/0	0/0	0/0	*e*

[a] The number of resistant isolates to each antibiotic with the shown chlorhexidine MIC.
[b] Antibiotic abbreviations: amikacin (AMI), amoxicillin/clavulanic acid (AUG), ampicillin (AMP), ceftiofur (TIO), ceftriaxone (AXO), cephalothin (CEP), chloramphenicol (CHL), ciprofloxacin (CIP), florfenicol (FFN), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET), and trimethoprim/sulfamethoxazole (COT).
[c] Number of antibiotic resistant and percent antibiotic resistant isolates with the listed chlorhexidine MICs, MIC = 0.47 μg/mL (N = 50), 0.94 μg/mL (N = 5), 1.88 μg/mL (N = 23), and 3.76 μg/mL (N = 11).
[d] These are the P values for the Mantel-Haenszel Chi-Square Test for the association of antibiotic resistance with chlorhexidine susceptibility for each antibiotic.
[e] Statistical evaluations were not made because some data points were zero.

Residual disinfectants may enter the environment in runoff or wastewater and can be found in surface and ground water, and in sediments and soils. There is concern about increasing resistant pathogenic bacteria in the environment because of low level antibiotic and disinfectant exposure (Kümmerer 2004). On-farm bacteria can also enter the human food chain in many ways. The food processing industry is a common portal, and is a location where biocide usage is high. Other ways for bacteria to enter the food chain are by discharge of waste to surface water, and the spreading of large amounts of animal waste on agricultural lands. The surface run-off and ground-water contamination by pathogens from intense animal production and waste lagoons is an emerging problem. We only need to remember the Walkerton, Ontario tragedy to put in perspective how contamination from manure can result in a large disaster (Ali 2004). This disaster occurred in a small Canadian farming community, where more than 2300 people became ill and seven people died because of *E. coli* O157:H7 contamination of well water (Bruce-Grey-Owen Sound Health Unit 2000). Also, there is a reported increased incidence of pathogens in the home environment, and it has been suggested that responsible use of biocides could contribute to reducing the impact of antibiotic resistance (Gilbert and McBain 2003).

The results presented in this paper demonstrate that a large percentage of these swine beta-hemolytic *E. coli* isolates exhibited consistent low-level chlorhexidine resistance. Reduced chlorhexidine susceptibility correlated with ribogroup, virulence factors, serotype, and with antibiotic resistance to gentamicin and streptomycin. The elucidation of resistance mechanisms, virulence factor involvement and the potential for transfer of this resistance is important. Environmental contamination with chlorhexidine and antibiotic resistant bacteria will increase the impact on resistance. Further surveillance is suggested for chlorhexidine and other disinfectants used in animal production that are also routinely used in human clinical settings and food production. Since chlorhexidine is an important biocide used both in veterinary and human medicine, it is of great importance to be aware of chlorhexidine resistance being linked to antibiotic resistance, and that bacterial isolates from animal production may be a source for this linked resistance.

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