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Klebsiella to *Salmonella* gene transfer within rumen protozoa: Implications for antibiotic resistance and rumen defaunation☆

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Abstract

The rumen has long been thought to be a site of gene transfer for microorganisms. Rumen protozoa (RPz) are active predators of bacteria that can harbor antibiotic resistance genes. In this study, RPz were assessed as sites of gene transfer between two bacterial species, *Salmonella* and *Klebsiella*. One *Klebsiella* isolate carried a plasmid bearing bla_{CMY-2}, encoding an extended-spectrum β-lactamase conferring ceftriaxone resistance, while the *Salmonella* was susceptible to ceftriaxone yet capable of thriving within protozoa. In vitro studies revealed that ceftriaxone-resistant *Salmonella* could be isolated following co-incubation of *Salmonella* and *Klebsiella* with RPz obtained from adult cattle and goats. Ceftriaxone-resistant *Salmonella* were not recovered in the presence of an inhibitor of protozoa engulfment or when a protozoa-sensitive *Salmonella* was part of the co-incubation. This transfer event was additionally observed in vitro for protozoa-independent stressors although at a significantly lower frequency. The gene transfer event was related to bacterial conjugation since a conjugation inhibitor, nalidixic acid, perturbed the phenomenon. Ceftriaxone-resistant *Salmonella* were recovered from calves, sheep, and goats co-challenged with ceftriaxone-resistant *Klebsiella* and ceftriaxone-sensitive *Salmonella*. However, the transfer event was not observed in calves and sheep that were defaunated prior to the co-challenge. Moreover, *Salmonella* transconjugants were isolated from separate bovine in vivo studies involving a *Klebsiella* donor carrying a plasmid conferring colicin activity while no such transconjugants were obtained from defaunated calves. These results provide an important basis for evaluating and preventing the spread of antibiotic resistance and other selective advantages for pathogens present in ruminants.
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Keywords: *Salmonella*; Conjugation; Protozoa; *Klebsiella pneumoniae*; Rumen; Antibiotic resistance; Ceftriaxone

☆ Mention of trade names of commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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1. Introduction

The microbial community of ruminant animals represents a diverse mix of prokaryotic and eukaryotic organisms interacting in complex metabolic pathways, representing a novel nutritional strategy for their animal hosts (Russell and Rychlik, 2001). In addition to these biochemical associations, there is opportunity for exchange at the genetic level. Gene transfer in the rumen is relevant given the mounting spread of antibiotic resistant bacteria and the public health implications for veterinary antimicrobial therapies. The first documentation of the transfer of antibiotic resistance between bacteria species in the rumen was reported over 25 years ago in sheep (Smith, 1975, 1977). Subsequent work has offered indirect evidence for gene transfer in ruminants, focusing either on the ability of rumen bacteria to acquire antibiotic resistance (Barbosa et al., 1999; Jonecova et al., 1994; Scott et al., 1997) or on the promotion of gene exchange by factors in the rumen environment (Fliegerova, 1993; Mizan et al., 2002; Scott and Flint, 1995). However, in vivo studies regarding pathogenic bacteria are lacking and the parameters and mechanism(s) of this phenomenon remain largely unexplored.

To date, the direct role of rumen protozoa (RPz) in antibiotic resistance gene transfer between bacteria has not been addressed. While in vitro studies have shown that engulfment by aquatic protozoa enhances bacterial conjugation (Schlimme et al., 1997), studies on the fate of bacteria in RPz are lacking. Differential uptake, as well as survival, of bacteria species by various RPz was initially described in a series of studies by G.S. Coleman (summarized in Dehority, 2003; Williams and Coleman, 1992) though it has generally been presumed that bacteria ingested by RPz succumb to digestive processes in protozoal vacuoles (Dehority, 2003). However, multiple studies have shown that *Salmonella* can survive within protozoa (Gaze et al., 2003; King et al., 1988; Tezcan-Merdol et al., 2004) including RPz (Rasmussen et al., 2005), which is notable given the potential for acquisition of antibiotic resistance genes in the rumen environment.

Herein, we investigated the role of RPz in bacterial gene transfer in the rumen. We identified and characterized the transfer of ceftriaxone resistance from a ceftriaxone-resistant *Klebsiella* isolate to a ceftriaxone-sensitive *Salmonella* recipient in the rumen. Co-incubations of these bacteria were conducted in the presence of RPz in vitro (bovine, caprine) and in vivo (bovine, caprine, and ovine). In order to determine whether the transfer event occurred independent of RPz, in vitro studies were conducted using disabled RPz and in vivo studies were conducted using defaunated animals. Experiments were also conducted in the absence of RPz in order to investigate the extent of the transfer event in conditions that were independent of RPz. Additional experiments examined the transfer of a colicin-encoding plasmid from *Klebsiella* to *Salmonella* in vivo.

2. Materials and methods

2.1. Strains used

All *Salmonella* strains (Table 1) were obtained from frozen stock cultures at the National Animal Disease Center (Ames, IA). For isolation and identification of the recipient *Salmonella* strains, GFP expression was conferred with the previously described pGFP plasmid containing a zeocin (Invitrogen) resistance marker (Frana and Carlson, 2001). *K. pneumoniae*, herein designated as TCR2003, and *K. ornithinolytica* (designated as TME2003) were isolated from turtle fecal samples collected in Iowa in 2003 and were identified using the BBL CRYSTAL Enteric/Nonfermenter ID System (Becton, Dickinson and Company, Sparks, MD) typing method. Ceftriaxone resistance in TCR2003 was examined by determining the MIC for ceftriaxone (breakpoint 64 μg/ml; Sigma) as per Clinical and Laboratory Standards Institute standards. Colicin activity, i.e., anti-*Escherichia coli* activity, in TME2003 was demonstrated using disk diffusion overlay and a zone of inhibition as described previously (Carlson et al., 2001) with TME2003 serving as the test strain and *E. coli* as the indicator strain. A colicin was putatively verified as the bacteriocidal agent by growing *E. coli* overnight in the presence of filter-sterilized TME2003 supernatant with or without the protease trypsin; there was no *E. coli* growth in the absence of trypsin (data not shown). TME2003 was also found to be