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Comparisons of *Salmonella* conjugation and virulence gene hyperexpression mediated by rumen protozoa from domestic and exotic ruminants

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ABSTRACT

Recent studies have identified a phenomenon in which ciliated protozoa engulf *Salmonella* and the intra-protozoal environment hyperactivates virulence gene expression and provides a venue for conjugal transfer of antibiotic resistance plasmids. The former observation is relegated to *Salmonella* bearing the SGI1 multiresistance integron while the latter phenomenon appears to be a more generalized event for recipient *Salmonella*. Our previous studies have assessed virulence gene hyperexpression only with protozoa from the bovine rumen while conjugal transfer has been demonstrated in rumen protozoa from cattle and goats. The present study examined virulence gene hyperexpression for *Salmonella* exposed to rumen protozoa obtained from cattle, sheep, goats, or two African ruminants (giraffe and bongo). Conjugal transfer was also assessed in these protozoa using *Salmonella* as the recipient. Virulence gene hyperexpression was only observed following exposure to the rumen protozoa from cattle and sheep while elevated virulence was also observed in these animals. Conjugal transfer events were, however, observed in all protozoa evaluated. It therefore appears that the protozoa-based hypervirulence is not universal to all ruminants while conjugal transfer is more ubiquitous.

1. Introduction

Salmonella enterica is a major cause of food borne illness throughout the world. Treatment of salmonellosis is becoming increasingly difficult as many stains are resistant to multiple antibiotics. This is especially true for *S. enterica* serotype Typhimurium phage type DT104 (DT104), which is resistant to five or more antibiotics as a result of the acquisition of an integron structure designated as *Salmonella* genomic island 1 [SGI1 (Briggs and Fratamico, 1999)].

Recent studies revealed that ciliated rumen protozoa (RPz), commensal microbes inhabiting the forestomach of ruminants, provide a venue for conjugal transfer where *Salmonella* can acquire plasmid-borne antibiotic resistance genes (McCuddin et al., 2006). Whereas many other bacteria are digested by RPz, *Salmonella* is an intracellular pathogen which is able to live, multiply, and accept conjugative plasmids within these microbes (McCuddin et al., 2006; Rasmussen et al., 2005).

In addition to mediating antibiotic resistance gene transfer, RPz hyperactivate *Salmonella* virulence in strains bearing the SGI1 integron. In this model, RPz engulf *Salmonella* and induce overexpression of *hilA*, a transcrip-

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tional regulator of invasion (Lee et al., 1992). HilA overexpression is in response to expression of SO13, an SGI1 gene with unknown function (Carlson et al., 2007a). The resulting hyperinvasion leads to a faster onset of clinical signs, a greater recovery rate of the pathogen, and a poorer prognosis. This is especially evident in cattle where DT104 is 13 times more likely to be lethal in calves when compared to calves infected with antibiotic-sensitive *S. enterica* serovar Typhimurium (Evans and Davies, 1996).

To assess this phenomenon in other ruminants, we assessed RPz isolated from goats, sheep, and two exotic ruminants (bongo and giraffe). RPz from these animals were evaluated for the ability to induce virulence gene hyperexpression and foster plasmid conjugation in *Salmonella*.

2. Materials and methods

2.1. Summary of microbes used in this study

Bacterial strains are summarized in Table 1 with strain 98-420 serving as the model DT104 isolate and strain TH11 serving as the DT104 isostrain lacking SGI1 (Carlson et al., 1999). Bacteria were stored in cryopreservation tubes containing 50% glycerol:50% culture medium at −80 °C and grown in LB broth (Sigma) without antibiotics for TH11, with ampicillin plus chloramphenicol (32 μg/ml each; Sigma) for DT104, or with ceftiofur (32 μg/ml; Pfizer) for *Klebsiella*.

2.2. Isolation of RPz for in vitro studies

2.2.1. Cattle, sheep, and goat RPz

Rumen fluid was removed from fistulated animals as described previously (Rasmussen et al., 2005). Fluid was filtered to remove large particulate matter and mixed with an equal volume of Coleman's buffer D (Coleman and Reynolds, 1982). Protozoa were then allowed to settle for 2 h under CO_2. Settled protozoa were aspirated and washed twice with approximately 45 ml Coleman's buffer D and then centrifuged for 20 s at 230 × *g*. Pelleted protozoa were resuspended in 30 ml Coleman's buffer D under CO_2. One milliliter was used for enumeration, and 3 ml (approximately 10^5 RPz) was used in each invasion assay. Genera of protozoa observed included *Eudiplodinium*, *Metadinium*, *Polyplastron*, *Isotricha*, *Entodinium*, *Ophryoscolex*, and *Diplodinium*.

2.2.2. African ruminant RPz

RPz were isolated from individually housed giraffe (*Giraffa camelopardalis*) and bongo (*Tragelaphus eurycerus*) at the Blank Park Zoo in Des Moines, IA, USA. Regurgitated rumen contents were collected from the surface of watering tanks and suspended in 300 ml Coleman's buffer. This solution was immediately filtered through cheese cloth to remove particulate matter and then centrifuged at 300 × *g* for 3 min. The pellet was resuspended in 10 ml of Coleman's buffer and a hemocytometer was used to determine an estimated RPz concentration of 10^5/ml. Trypan blue staining revealed approximately 90% viability of the RPz, which is consistent with previous studies employing this method of RPz sampling (Ellis et al., 1989). Control studies revealed that this sampling method yielded viable bovine RPz that facilitated plasmid transfer and hyperinvasion (data not shown). Additionally, the water at the Blank Park Zoo did not contain any free-living environmental protozoa.

2.3. Salmonella invasion assays following survival within RPz

10^5 RPz were co-incubated with 2×10^8 CFUs *Salmonella* and agitated with a tube roller for 24 h at room temperature in a 1.5 ml microcentrifuge tube. Extracellular *Salmonella* were then killed using a super-MIC/super-MBC concentration (300 μg/ml) of florfenicol (Schering-Plough) for 2 h. RPz were then lysed for 60 s at 4800 rpm using 2.5 mM glass beads and a mini-beadbeater (Biospec Products). The recovered lysate was pelleted and resuspended in 350 μl LB broth and 50 μl was plated on XLT agar (Difco) to assess engulfment of *Salmonella*. The remaining bacteria were incubated with HEp-2 cell cultures in triplicate. Specifically, 100 μl (approximately 10^6 CFUs) was added to each tissue culture well containing 10^5 HEp-2 cells which were maintained in RPMI 1640 (GIBCO) containing 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified incubator. The protozoa-derived bacteria were incubated with HEp-2 cells for 1 h at 37 °C and extracellular bacteria were then killed using 300 μg/ml florfenicol for 2 h. HEp-2 cells were then lysed for 15 min at 37 °C using 0.1% Triton-X. Dilutions of the lysate were plated on *Salmonella* selective media and grown overnight at 37 °C followed by enumeration the next day. Percent invasion was calculated by dividing CFUs recovered by CFUs added.

2.4. Conjugation frequency assay

Approximately 10^9 CFUs of both *S. enterica* serotype Typhimurium strain TH11 (Carlson et al., 1999) and *K. pneumoniae* TCR2003 (McCuddin et al., 2006) were co-incubated with approximately 10^5 RPz in sealed glass tubes overnight on a rocker at 37 °C. At the end of the incubation period, extracellular *Salmonella* were killed using 300 μg/ml

Table 1
Summary of microbes used in this study.

Microbe	Relevant characteristics	Reference
Salmonella enterica serotype Typhimurium DT104 strain 98-420	SGI1-bearing strain capable of exhibiting hypervirulence following exposure to RPz; florfenicol MIC equals 64 μg/ml	Rasmussen et al. (2005)
Salmonella enterica serotype Typhimurium DT104 strain TH11	SGI1-free isostrain of DT104 incapable of hyperinvasion	Carlson et al. (1999)
Klebsiella pneumoniae TCR2003	Isolated from turtle feces in Iowa; bears a ceftiofur resistance plasmid; ceftiofur MIC equals 64 μg/ml	McCuddin et al. (2006)

florfenicol. RPz were then lysed using a bead beater for 60 s. The lysate was pelleted and resuspended in 200 ml Lennox L broth. Next, 50 μl was spread on Brilliant Green Agar (does not support the growth of *Klebsiella*; Difco) containing 32 μg/ml ceftiofur. Plates were incubated at 37 °C overnight and colonies were enumerated the next day. Conjugation frequency was determined by calculating CFUs of ceftiofur-resistant *Salmonella* recovered divided by CFUs of ceftiofur-sensitive *Salmonella* added to the assay.

2.5. In vivo infection experiments

All animals were assessed for the lack of fecal shedding of *Salmonella* via PCR (Carlson et al., 1999). Additionally, all animals were deemed to be seronegative for anti-*Salmonella* antibodies via an ELISA assay (Stabel et al., 1995). 10^6 CFUs/kg of DT104 was orally inoculated into 8-week-old Holstein calves ($n = 6$, approximately 70 kg each, male), adult sheep ($n = 6$, approximately 100 kg each, female, mixed breeds), and goats ($n = 6$, approximately 100 kg each, female, mixed breeds) which harbored native populations of RPz. Animals were monitored for changes in appetite, stool consistency, and rectal temperature every 8–12 h. At the onset of signs related to systemic salmonellosis, animals were euthanized using xylazine (0.5 mg/kg intramuscularly; Phoenix Laboratories) and pentobarbital (1.2 mg/kg, intravenously; Fort Dodge Laboratories). Splenic samples, which are a reliable indicator of systemic burden (Carlson et al., 2007a; Rasmussen et al., 2005), were collected following euthanasia. Animal experiments were approved by the Animal Care and Use Committee at the National Animal Disease Center [former employer of S.A.C. (protocol 3462)].

2.6. Quantitation of Salmonella systemic burden

Spleens were aseptically cut into small pieces, placed in an equal volume of LB broth, and vortexed vigorously for 2 min. Dilutions of the resulting homogenate were plated on XLT agar containing ampicillin (32 μg/ml) and chloramphenicol (32 μg/ml) for enumeration. *Salmonella* colonies were confirmed by PCR using primers specific for detection of the *sipB-sipC* gene fragment as described previously (Carlson et al., 1999).

2.7. Semi-quantitative RT-PCR

RT-PCR was conducted to assess the expression of *hilA* and SO13. Bacteria were isolated from RPz on agar and then RNA was isolated from 10^6 CFUs of bacteria using the RNeasy Mini Kit with enzymatic lysis for the initial cell disruption (Qiagen). RT-PCR was carried out using approximately 0.5 μg of total RNA, the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen), the *hilA* or SO13 primers, and PCR conditions described previously (Carlson et al., 2007a). The number of cycles in the PCR portion of the reaction was varied to empirically determine the number of cycles required to produce a visible amplicon from the *Salmonella* RNA template collected from each specimen. RT-PCR products were visualized on a 1.5% percent agarose gel using ethidium bromide staining.

3. Results

3.1. Assessment of Klebsiella to Salmonella conjugal transfer of antibiotic resistance plasmids within RPz from various ruminants

Our previous studies revealed that *Salmonella* will conjugate and receive antibiotic resistance plasmids while co-habitating in RPz obtained from cattle and goats. This previous study used a *Klebsiella* donor strain that contains a plasmid that is only transferred to *Salmonella* when the *Klebsiella* is stressed (McCuddin et al., 2006). To determine if this phenomenon extends beyond these two domestic ruminants, this *Klebsiella* isolate was co-incubated with recipient *Salmonella* in the presence of RPz obtained from sheep and two African ruminants. The giraffe was chosen because a previous study demonstrated that these ruminants have unique RPz (Kleynhans and van Hoven, 1976; Dehority and Odenyo, 2003). The bongo was chosen since it is a member of the Bovidae family and thus their RPz may behave like cattle RPz.

Conjugation was measured by enumerating recovered ceftiofur-resistant *Salmonella* since the *Klebsiella* bears a plasmid encoding an extended-spectrum beta-lactamase (McCuddin et al., 2006). As shown in Table 2, the *Klebsiella*-to-*Salmonella* plasmid transfer events were also observed in RPz obtained from sheep, giraffe and bongo. Conjugation frequencies were slightly higher in RPz obtained from the African ruminants although this difference may not be biologically significant.

3.2. Assessment of DT104 hyperinvasion after exposure to RPz obtained from various ruminants

Our previous studies revealed that SGI1-bearing strains of *Salmonella* are hyperinvasive after exposure to RPz

Table 2
Summary of *Klebsiella-Salmonella* gene transfer events observed *in vitro*. *K. pneumoniae* TCR2003 was used as the donor strain in all studies. Experiments used 10^9 potential recipients, 10^9 donors, and 10^4 RPz.

Salmonella recipient	RPz source	Replicates	Number of ceftiofur-resistant *Salmonella* recovered/10^9 recipients (mean ± SEM)
TH11	Bovine	12	408 ± 85
TH11	Caprine	12	387 ± 115
TH11	Ovine	12	298 ± 37
TH11	Bongo	12	877 ± 83
TH11	Giraffe	12	1094 ± 27
TH11	No RPz	12	0

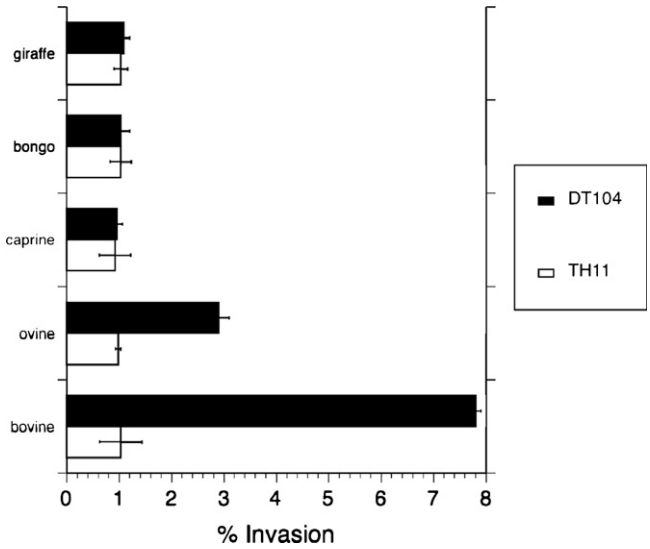

Fig. 1. Assessment of HEp-2 cell invasion by SGI1-bearing (DT104) or SGI1-free (TH11) *Salmonella* recovered from RPz. The source of the RPz is indicated on the *y*-axis. Each bar represents the mean ± standard error for 12 replicates.

obtained from cattle. To determine if this phenomenon extends beyond cattle, SGI1-bearing *Salmonella* were evaluated for ovine and caprine RPz-mediated alterations in invasiveness using a HEp-2 tissue culture cell invasion assay. Additionally, we assessed this phenomenon in RPz obtained from the bongo and giraffe. The hyperinvasive state of DT104 was compared to that of TH11, a strain that lacks SGI1 and therefore is not capable of exhibiting the RPz-mediated hyperinvasive phenotype (Carlson et al., 2007a; Rasmussen et al., 2005). Since free-living protozoa can induce DT104 hyperinvasion (Carlson et al., 2007a), these results indicate that the bongo and giraffe samples contained mostly RPz.

As shown in Fig. 1, DT104 invasion was increased following recovery from bovine RPz which is consistent with previous data (Carlson et al., 2007a; Rasmussen et al., 2005). DT104 recovered from ovine RPz were also significantly more invasive when compared to that observed for TH11 although the increase in invasiveness was not as robust as the increase noted in the presence of

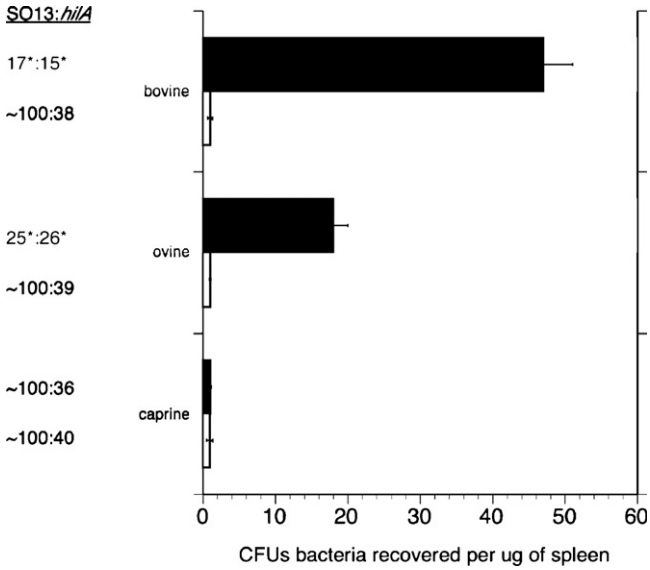

Fig. 2. Assessment of bacterial loads and semi-quantitative analysis of virulence gene expression for *Salmonella* DT104 or TH11 exposed to RPz in sheep, calves, and goats. Left side, virulence gene expression of *hilA* and SO13 in DT104 (upper sets of numbers) and TH11 (lower sets of numbers) recovered from calves, sheep, and goats. Values indicate the number of PCR cycles necessary to generate a visible SO13 or *hilA* amplicon following an RT-PCR assay. Right side, quantitation of DT104 (filled bars) or TH11 (open bars) recovered from splenic samples of animals infected with the pathogens. Each bar represents the mean ± SEM for 6 animals.

bovine RPz. For all other RPz examined, invasiveness was indistinguishable for DT104 and TH11.

3.3. Assessment of RPz-mediated Salmonella hypervirulence in vivo

Previous studies indicated that RPz-mediated hyperinvasion is a predictor of hypervirulence *in vivo* (Carlson et al., 2007a; Rasmussen et al., 2005). In the present study we assessed clinical outcomes in calves, sheep, and goats (negative control) infected with DT104 exposed to RPz. Studies involved monitoring the clinical signs and culturing splenic samples from infected animals. Control animals were infected with TH11 lacking SGI1 (Carlson et al., 2007a; Rasmussen et al., 2005).

As depicted in Fig. 2, significantly more DT104 were recovered from calves and sheep when compared to goats. Similarly, calves and sheep demonstrated pyrexia 24–48 h post-infection whereas goat rectal temperatures did not differ from normal (Fig. 3).

3.4. Assessment of SO13 and hilA expression in DT104 inoculated into cattle and sheep

Our previous studies indicated that RPz-mediated hypervirulence in DT104 is associated with enhanced expression of *hilA* and SO13 following protozoal engulfment (Carlson et al., 2007a). In the present study, a semi-quantitative RT-PCR was used to determine relative expression levels of *hilA* and SO13 in DT104 and TH11 recovered from calves, sheep, and goats.

As shown in Fig. 2, RPz-mediated hypervirulence was associated with increased expression of *hilA* and SO13 in DT104 recovered from calves and sheep. This was especially evident for the SO13 amplicon which could be amplified from DT104 recovered from calves and sheep in the primary RT-PCR reaction. In contrast, secondary and tertiary PCR reactions were required before a visible amplicon could be generated in TH11 recovered from all animals and from DT104 recovered from goats.

4. Discussion

Previously we have demonstrated that DT104 exhibits hypervirulence following exposure to RPz obtained from cattle (Carlson et al., 2007a; Rasmussen et al., 2005). Additionally, bovine and caprine RPz can serve as venues for plasmid acquisition by *Salmonella* (McCuddin et al., 2006). The study herein describes experiments addressing the hypothesis that these phenomena extend to an array of RPz obtained from domestic and exotic ruminants although the exotic ruminants were housed in a domestic environment.

Plasmid transfer studies revealed that all RPz tested could serve as facilitators for this event. This finding is consistent with our unpublished observations that free-living protozoa, such as *Tetrahymena* and *Acanthamoeba*, can mediate plasmid transfer into *Salmonella*. Additionally, our preliminary studies suggest that *Salmonella* can acquire antibiotic resistance genes while residing within pathogenic *Eimeria* and *Trichomonas* (not shown). That is, *Salmonella* appears to be able to conjugate with other bacteria in a wide array of protozoa. Based on our previous studies, it is unclear if this conjugation process can occur within mammalian cells that engulf or are passively invaded by *Salmonella* (McCuddin et al., 2006). Differences in intracellular trafficking may account for this divergence.

The instigation of hypervirulence, however, is not as ubiquitous. Hyperinvasion and hypervirulence were noted only for *Salmonella* exposed to RPz obtained from cattle and sheep. Expression of SO13 and hyperexpression of *hilA* were associated with the augmented invasion and virulence observed in sheep in a manner similar to our previous (Carlson et al., 2007a) and current findings with cattle. There does appear to be a difference in the clinical progression of hypervirulence in cattle versus sheep, while the disease course in goats was unremarkable. We hypothesize that differences in RPz profiles account for the non-conserved nature of the hyperinvasion/hypervirulence. *Isotricha* is a putative non-engulfing RPz (Dehority and Odenyo, 2003; Rasmussen et al., 2005) that may be

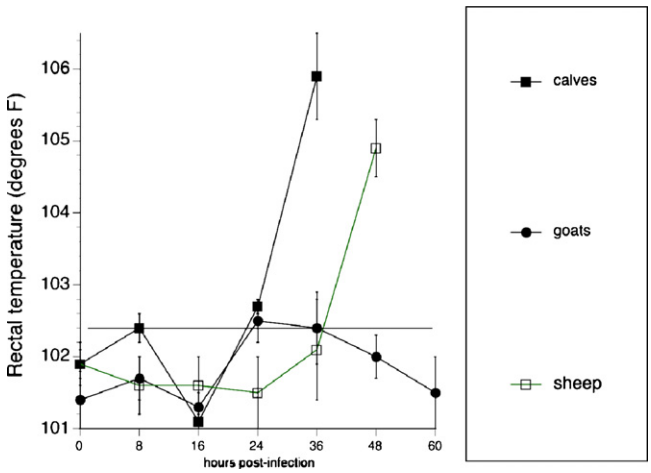

Fig. 3. Onset of pyrexia in calves, sheep, and goats orally infected with DT104. Rectal temperatures were determined every 8–12 h. The upper limit for normal bovine rectal temperature (~102.5) is indicated by the horizontal line. Each point represents the mean ± SEM for 6 animals.

relatively less abundant in the bovine and ovine rumens. Alternatively, sheep and cattle rumens may contain unique RPz that engulf and modulate gene expression in *Salmonella*. The time of day (Ankrah et al., 1990), time after feeding (Nakamura and Kanegasaki, 1969; Purser and Moir, 1966), and diet (Abe et al., 1973) are other factors influencing RPz profiles. It is also possible that cattle RPz harbor unique bacteria that facilitate the observed phenomenon regarding hypervirulence.

In summary, the phenomenon of RPz-mediated *Salmonella* gene transfer is viable in cattle, sheep, goats, bongo, and giraffe. The RPz-hypervirulence phenomenon is relegated to cattle and sheep. RPz profiles are the likely determinant of the divergence regarding the latter phenomenon.

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