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In Vitro Characterization and *In Vivo* Properties of Salmonellae Lytic Bacteriophages Isolated from Free-Range Layers

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ABSTRACT

Occurrence of food poisoning related to *Salmonella*-contaminated eggs and chicken meat has been frequent in humans. *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST) are included among the most important paratyphoid salmonellae associated with chicken meat and eggs. Elimination of *Salmonella* at the pre-harvest stage can play a significant role in preventing the introduction of this pathogen into the food chain and consequently in the reduction of food poisoning in humans. Bactericidal bacteriophages may provide a natural, nontoxic, feasible and non-expensive component of the multi-factorial approach for a pre-harvest control of *Salmonella* in poultry. Five bacteriophages lytic for SE PT4 and ST were obtained from 107 samples of feces of free-range layers in Brazil. All bacteriophages were characterized *in vitro* and *in vivo*, showing head and tail morphology and dsDNA as nucleic acids. Results of *in vivo* studies suggested that bacteriophages do not remain in *Salmonella*-free birds longer than one day, whereas they multiply in *Salmonella*-infected birds for longer periods. Besides, selection for phage-resistant SE PT4 did not seem to occur in the short term. Isolated bacteriophages will be investigated for their potential for pre-harvest biocontrol of SE PT4 in poultry.

INTRODUCTION

Occurrence of food poisoning related to *Salmonella*-contaminated eggs and chicken meat has been frequent in humans. Food poisoning caused by *Salmonella* results in reduction of productivity, discomfort, expenditures with medication and eventually death (Persson & Jendteg, 1992; Mead *et al.*, 1999). Eating raw or undercooked eggs has also been considered a major risk factor for food poisoning with salmonellae in some situations (Molback & Neiman, 2002). *Salmonella* Enteritidis (SE) (Tavechio *et al.*, 2002; Chung *et al.*, 2003) and *Salmonella* Typhimurium (ST) (Flensburg 1999; Taunay *et al.*, 1996) figure among the most important paratyphoid salmonellae associated with chicken meat and eggs.

SE is the most frequently isolated *Salmonella* from poultry products in Brazil. Santos *et al.* (2000) reported up to 53.5% of frozen broilers as being contaminated by *Salmonella*, so that SE represented 60% of total isolates and dos Santos *et al.* (2003) reported that 57.65% of SE isolated in Brazil were of phage type 4 (SE PT4). Between 1993 and 1997, Laboratory I from Instituto Adolfo Lutz (São José do Rio Preto, São Paulo, Brazil) identified that raw eggs were involved in 95.7% of all food poisoning cases caused by SE PT4 (Paresi *et al.*, 1998). SE was also the most prevalent; it represented 32.7% of 4,581 *Salmonella* strains isolated from non-human sources since January 1996 until December 2000, at Instituto Adolfo Lutz, São Paulo, Brazil (Tavechio *et al.*, 2002). On the

other hand, the percentage of ST isolation in food poisoning cases decreased from 77% in the 1970's to 36% in the 1980's (Taunay *et al.*, 1996). However, the occurrence of ST in poultry products in Brazil and other countries (Flensburg, 1999) still demands efforts of the poultry industry to reduce its occurrence.

Elimination of *Salmonella* at the pre-harvest stage can play a significant role in preventing the introduction of this pathogen into the food chain and consequently in the reduction of food poisoning in humans (Wegener *et al.*, 2003). Pre-harvest control of *Salmonella* is more likely to be effective through a multi-factorial approach. Good agricultural practices such as hazard analysis and critical control point (HACCP) (Rose *et al.*, 2002; Nayak *et al.*, 2003), vaccination (Cogan & Humphrey, 2003; Zhang-Barber *et al.*, 1999; Yamame *et al.*, 2000), probiotics, prebiotics and synbiotics (Van Immerseel *et al.*, 2002) have been used as preventive measures when *Salmonella* infection is likely to occur. However, the use of anti-microbial drugs many times is still required when other measures are not efficient enough (Davies *et al.*, 2003; Fernadez *et al.*, 2001). In such cases, a new concern may arise due to the selection of resistant bacteria (Chung *et al.*, 2003; Molbak *et al.*, 2002; Velonakis *et al.*, 2001) or the possibility that drug residues remain in meat and eggs consumed by humans (Donoghue, 2003).

In order to reduce the use of anti-microbial drugs, new methods for *Salmonella* control within the poultry production are required to be used in conjunction with good agricultural practices. These methods must be efficient in reducing *Salmonella* contamination on the final product without increasing production costs or introducing risks of chemical contamination of poultry products. Bactericidal bacteriophages may provide a natural, nontoxic, feasible and non-expensive component of the multi-factorial approach for a pre-harvest control of *Salmonella* in poultry. In this study, bacteriophages were isolated using SE PT4 and ST as bacterial targets and were evaluated for morphology and ability to lyse cultures *in vitro*. Unique profiles of DNA amplification were identified. Three bacteriophages were administered to SE-infected and non-infected Specific Pathogen Free (SPF) chickens to study the dynamics of their excretion.

MATERIAL E METHODS

Bacterial strains

SE PT4 isolate P125589 was kindly provided by Dr Paul Barrow, from the ARFC Institute for Animal Health, Houghton Laboratory, Cambridge, England (Barrow & Lovell, 1991). Dr B. Rowe originally obtained this isolate from a case of human food poisoning at the Central Public Health Laboratory, London, UK. ST was purchased from the American Type Tissue Collection (Manassas, Virginia, USA), where it is deposited under the number 14028 as *Salmonella choleraesuis* subsp. *choleraesuis* serotype Typhimurium. Salmonellae were cultivated in Nutrient Agar or Broth (NA or NB, 1g/L beef extract, 2g/L yeast extract, 5g/L peptone, 5g/L sodium chloride, and 15g/L bacteriologic agar for solid media) and kept frozen at –80°C unless stated.

Bacteriophage isolation from feces.

A total of 107 feces samples from individuals of different avian species (Table 1) were collected from the ground and subjected to bacteriophage isolation under the technique described by Kudva *et al.* (1999). Briefly, one gram of feces was inoculated into 10mL of a log phase SE PT4 or ST culture grown in nutrient broth added with 5mM $MgSO_4$ (NB-$MgSO_4$). This mixture was incubated for approximately 18 hours at 37°C under shaking (200rpm) and treated with 5% chloroform to lyse the bacteria. The preparation was centrifuged (12,000 X g for 5 minutes) and 10µL of supernatant was applied onto an overlay culture of *Salmonella*. Overlays were prepared by inoculation of 250mL of a *Salmonella* culture in exponential growth into 7mL of melted NB (45°C) containing 0.7% agarose. The melted agarose containing the bacteria was laid over a 10cm-diameter sterile Petri dish with nutrient agar and incubated for 24 hours at 37°C after solidification. The plaques of inhibition of *Salmonella* growth on the agarose layer, corresponding to lyse caused by bacteriophages, were cut for cloning.

Table 1 - Samples subjected to bacteriophage isolation.

Birds	Samples	Location
Free range layers	40	Concórdia, SC, Brazil
Broiler breeders	45	Concórdia, SC, Brazil
Rhea	13	Venâncio Aires, RS, Brazil
Caged songbirds	9 pools	Concórdia, SC, Brazil
Total	107	-

The cut agarose was vortexed and diluted tenfold in SM buffer (5.8g/L NaCl, 2g/L $MgSO_4.7H_2O$, 0.05M Tris-HCl pH7.5, 0.1g/L gelatin) and 10µL of each dilution were inoculated into 250mL of a log phase *Salmonella* culture. After 20 minutes at 37°C, it was inoculated into 7mL of NB with 0.7% agarose and overlay cultures

were prepared as described above. This procedure was repeated three times with isolated plaques to purify the cultures. Final clones were named, amplified on 50mL of a log-phase *Salmonella* culture and stored at -80°C in SM buffer with 7% Dimethyl Sulfoxide (DMSO) and 1% chloroform or at 4°C as a supernatant containing 1% chloroform.

Salmonella isolation

One gram of feces was inoculated into 9mL of Rappaport-Vassiliadis Soya Peptone broth (RVS, 4.5g/L soya peptone, 7.2g sodium chloride, 1.26g/L potassium dihydrogen phosphate, 0.18g/L di-potassium hydrogen phosphate, 13.58g/L magnesium chloride anhydrous, 0.036g/L malachite green, pH5.2) and incubated at 42°C for 24 hours. A loopful was then transferred to Brilliant Green Agar plates (BGA, 10g/L proteose peptone, 3g/L yeast extract, 10g/L lactose, 10g/L sucrose, 5g/L sodium chloride, 0.08g/L phenol red, 0.0125g/L brilliant green, 12g/L bacteriologic agar, pH 6.9) and incubated at 37°C for 48 hours. Light pink-white opaque colonies surrounded by red medium were tested by agglutination with polyvalent anti-somatic serum.

Lytic capability

Capacity of lysis was assessed for all bacteriophages using routine test dilution (RTD). Bacteriophage stocks were logarithmically diluted tenfold and aliquots of 50µL dropped onto ST or SE PT4 overlay cultures immediately after agarose solidification and incubated at 37°C for 24 hours. The highest dilution that caused confluent lysis of bacteria on the *Salmonella* overlay was recorded and compared among the different bacteriophages.

Electron microscopy

Electron microscopy was performed with standard techniques (Hayat, 1989) on bacteriophage-inoculated *Salmonella* cultures. Each bacteriophage was inoculated from fresh stocks into 1.5mL of a log phase culture of the same *Salmonella* used for its isolation and the culture kept for 6 hours at 37°C under shaking (200rpm). Bacteriophage infected-bacteria were collected by centrifugation (12,000 X g for 5 minutes) and the pellet was embedded in 100µL of 4% agarose. The pellet was cut in small fragments and fixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1M cacodylate buffer (pH7.4), postfixed in osmium tetroxide, dehydrated in ascending series of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranil acetate and lead citrate and examined using an EM 109 Zeiss transmission electron microscope at 80 kW.

DNA analysis

Bacteriophages were produced in large scale by inoculating overlay cultures prepared on four 15cm-diameter Petri dishes. Inoculations were performed to yield confluent plaques after incubation for 24 hours at 37°C. Bacteriophage particles were collected by overnight diffusion into 50mL of SM buffer laid on top of agarose, which was treated with 5% chloroform and centrifuged (12,000 X g for 10 minutes at 4°C) to remove bacterial debris. Bacterial nucleic acids were removed by digestion with 1mg/mL of DNAse 1 and RNAse A for three hours at 37°C. Bacteriophages were then precipitated overnight at 4°C with 2% PEG 8000 and 2.5M sodium chloride and collected by centrifugation (12,000 X g for 15 minutes at 4°C) (Helms *et al.*, 1987). Bacteriophage nucleic acids were purified by standard phenol extraction and ethanol precipitation (Sambrook *et al.*, 1989) and used for nuclease digestion tests or Random Amplified Polymorphic DNA Analyses (RAPD).

Approximately one microgram of bacteriophage nucleic acids were reacted for one hour at 37°C with 5IU of DNAse 1, Nuclease S1 or RNAse A diluted in appropriate buffers. Reacted nucleic acids were subjected to electrophoresis (110V/1:30h) in 1% agarose in TE buffer (10mM Tris-HCl, 1mM EDTA, pH7.5) gels stained with ethidium bromide (10mg/mL) to identify possible degradation caused by the enzymes.

RAPD was performed with the "Ready-To-Go RAPD Analysis Beads" (Amersham Pharmacia Biotech) with all six primers in individual reactions. Amplification was standardized with 25ng DNA and 25pmol primers added to the components present in the reaction tube and a final volume of 25mL. Reactions were performed with 45 cycles of 95°C for one minute, 36°C for one minute and 72°C for two minutes. Amplified fragments were separated on 2% agarose gels electrophoresis as described above.

In vivo bacteriophage properties

The three bacteriophages denominated CNPSA1, CNPSA3 and CNPSA4 (Table 2) were selected and administered to birds because their RAPD profiles indicated they were distinct viruses. Three groups with seven specific pathogen free (SPF) chickens (SPAFAS, Charles River Laboratories) were housed in isolator cabinets with filtered air from one until 16 days of age. Group 1 was orally infected at the third day of age

with 10^3 colony-forming units (CFU) of SE PT4 per bird. Two days later, each bird of Group 1 was orally inoculated with a pool of 10^5 plaque forming units (PFU) of each phage. Group 2 was inoculated only with bacteriophages, at the same dose, and Group 3 was kept as uninfected control (Table 3).

Table 2 – Isolated bacteriophages.

Bacteriophage	Origin of feces	Bacterium used as a target
CNPSA 1	Free range layer, Concórdia, SC	SE PT4
CNPSA 2	Free range layer, Concórdia, SC	ST
CNPSA 3	Free range layer, Concórdia, SC	ST
CNPSA 4	Free range layer, Concórdia, SC	ST
CNPSA 5	Pool of captive songbirds, Concórdia, SC	ST

Table 3 – Experimental design for in vivo study of bacteriophage isolates.

Group	Birds	SE PT4 per bird	Bacteriophages
1	7	10^3 CFU at the 3rd day	10^5 PFU at the 5th day
2	7	None	10^5 PFU at the 5th day
3	7	None	None

Cloacal swabs were collected daily for SE PT4 and bacteriophage isolation. *Salmonella* was isolated by incubating swabs overnight at 42°C in 3mL of RVS, followed by loopful inoculations on BGA and incubation at 37°C for 48 hours. Identification of *Salmonella* was carried out by colony morphology and serum agglutination as described above. Bacteriophages were isolated by incubating swabs at 37 °C in one milliliter of an exponential-phase culture of SE PT4. After treatment with 5% chloroform and centrifugation (12,000 X g for 5 minutes), 5µL of the supernatant were inoculated onto a overlay of SE PT4 grown on NB-$MgSO_4$ solidified with 0.7% agarose. Presence of bacteriophages was identified by transparency of the bacterial overlay on sample sites.

At the 16th day of life, all birds were necropsied and attempts were made to isolate SE PT4 and bacteriophages from the ceca, spleen and liver of every bird. Fragments of tissue used for *Salmonella* isolation were inoculated in 3mL of RVS followed by inoculation in BGA as described above. Attempts to isolate bacteriophage were made with fragments of tissue cultivated in 1mL of SE PT4 in exponential growth followed by treatment with 5% chloroform, centrifugation and inoculation of 10µL of supernatant onto bacterial overlays as described above.

Five isolates of *Salmonella* were obtained from the ceca of each bird from the group that was inoculated with SE PT4 and bacteriophage. Bacteriophage sensitivity was assessed for all isolates. Five microliters of each bacteriophage stock was applied onto a overlay of the *Salmonella* isolates grown on NB solidified with 0.7% agarose.

Cecal contents obtained at necropsy were weighed and logarithmically diluted tenfold in PBS pH7.2. Dilutions (100µL) were inoculated on BGA with Novobiocin (40 µg/mL) to obtain CFU/g.

RESULTS AND DISCUSSION

Bacteriophage isolation

Five bacteriophages were isolated from 107 (4.67%) tested samples. Bacteriophages were denominated CNPSA1, CNPSA2, CNPSA3, CNPSA4, and CNPSA5, the letters stand for the name of the research institute and the number is the order of bacteriophage isolation. CNPSA5, which was isolated from captive birds, was the only bacteriophage obtained from a source other than free-range layers. Probably, the broad range of feed sources naturally available to free-range chickens and their freedom to move in a larger area had some influence in the success of bacteriophage isolation. Bacteriophages lytic to *Salmonella* have been isolated from sewage water and poultry litter (Berchieri Jr *et al.*, 1991; Sklar *et al.*, 2001), demonstrating their natural occurrence in the environment.

Bacteriophage CNPSA2 could not be recovered after a few weeks stored either at 4°C or -80°C and was therefore not included in additional studies. It was not possible to identify a reason for the loss of viability, but once all bacteriophages were submitted to the same management same, it is more likely that it was related to the nature of CNPSA2 instead of laboratory handling.

All bacteriophages produced small clear and round plaques with well-defined edges and ranging from 1 to 1.5mm of diameter, in both salmonellae tested. This is an indication that all bacteriophages are lytic, however the possibility that they have a lysogenic stage cannot be ruled out, once specific tests for lysogeny were not carried out.

Salmonella isolation from field samples

No *Salmonella* was isolated from the 107 feces samples. The fact that bacteriophage-positive feces were negative in attempts to isolate *Salmonella* cannot be fully explained. The feces were collected from the ground and may have been contaminated by

bacteriophages from the environment. Also, all bacteriophages were isolated from samples obtained from adult free range chickens or captive songbirds, which are likely to be subjected to a mild *Salmonella* infection and therefore shed very low numbers of CFU in the feces (Asheg *et al.*, 2001). Low shedding of *Salmonella* may not be detected in conventional bacteriologic tests due to overgrow of other bacteria when inoculated into RVS. Bacteriophages, however, last for longer periods in the environment and are eliminated in larger numbers. This makes bacteriophages more likely to be recovered when shed in the feces along with *Salmonella*, because they are amplified in the SE PT4 culture inoculated with feces.

Lytic capability

RTD assays demonstrated that all bacteriophages lyse both SE PT4 and ST at similar multiplicity of infection (MOI). Confluent lysis was obtained with bacteriophage stocks containing similar PFU titers diluted as high as 10^{-3} and 10^{-4} when 50µL were applied onto overlay cultures of either *Salmonella*. These results indicated that capacity of lysis *in vitro* could not be a criterium for selection of bacteriophages to be used in biocontrol of *Salmonella*. MOI also could not be used as a standard to express bacteriophage infectivity.

Electron microscopy and DNA analysis

All isolated bacteriophages showed similar head and tail ultrastructure (Figure 1) ranging from 80 to 120mm. Head and tail ultra-structure is a common feature of the morphology of bacteriophages (Levine, 1992). Some microscopy fields showed bacteriophages inside *Salmonella* cell debris (Figure 2), confirming their capacity to lyse *Salmonella* cells.

Bacteriophages were identified as dsDNA viruses in the assays carried out with bacteriophage nucleic acids and the different nucleases. Digestions with DNAse 1 caused degradation of nucleic acids while electrophoretograms showed that their nucleic acids reacted with Nuclease S1, which cleaves ssDNA. Besides, treatment with RNAse A showed the expected bands of non-digested DNA. Families of tailed bacterial viruses with dsDNA are Myoviridae, Podoviridae and Siphoviridae (ICVT, 2003).

RAPD allowed differentiation of all bacteriophages but for CNPSA4 and CNPSA5 (Figure 3). Since only six primers were tested, we cannot rule out the possibility that these two isolates may actually represent different bacteriophages. In order not to test twice the same bacteriophage, CNPSA5 was not evaluated *in vivo*.

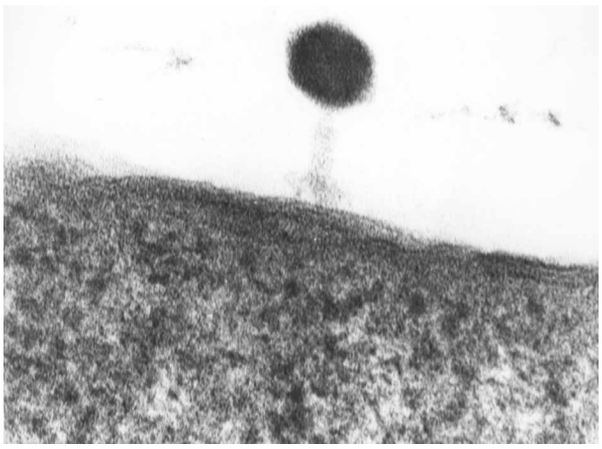

Figure 1 - Bacteriophage showing the characteristic ultra-structure of head and tail with tail fibers inserted into Salmonella Enteritidis membrane. 238,000x.

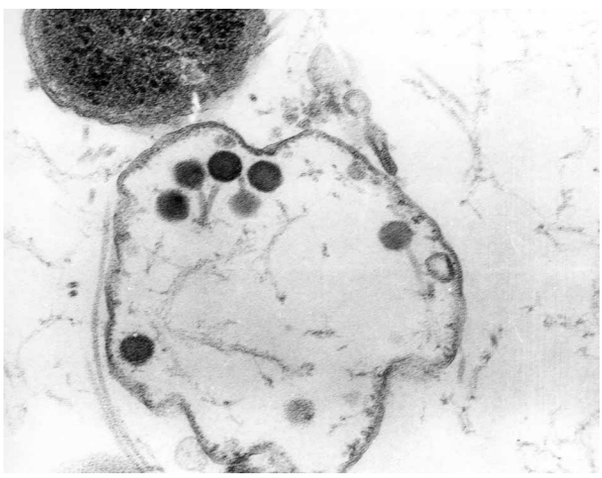

Figure 2 - Bacteriophage particles inside bacterial debris. 86,000x

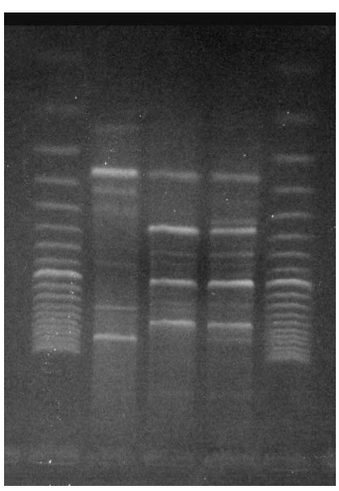

Figure 3 - RAPD profiles showing differences between bacteriophages used for in vivo characterization. Lanes from left to right: 100bp ladder, DNA of bacteriophages CNPSA1, CNPSA3, CNPSA4, CNPSA5, 100bp ladder. DNA of bacteriophages was amplified with the primer 5'-d[GGTGCGGGAA]-3'. Bacteriophage CNPSA2 lost viability in the laboratory.

RAPD proved to be a useful tool to check purity of cultures and to confirm identity of isolates in clinical trials.

In vivo properties

Cloacal swabs of control birds (Group 3) were negative for both bacteriophages and *Salmonella* throughout the experiment. Swabs collected from internal organs of control birds were also negative for both bacteriophages and *Salmonella*, confirming the negative status of the control group for *Salmonella* and the non-occurrence of bacteriophages either in the SPF chickens or in the feed used in the experiment.

SE PT4 uninfected-phage administered birds (Group 2) showed only one bacteriophage-positive cloacal swab out of seven swabs collected one day after bacteriophage administration, and all swabs were negative thereafter. All seven swabs of SE PT4 infected-phage administered chickens (Group 1) were positive for the presence of bacteriophage two days after administration, ranging between 4/7 and 7/7 thereafter (Figure 4).

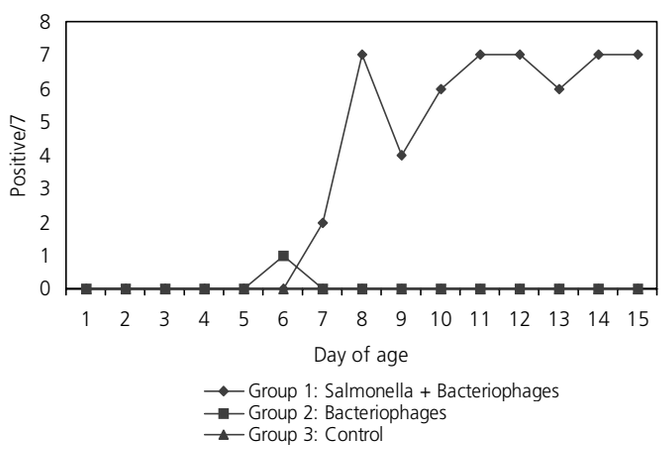

Figure 4. Frequency of bacteriophage isolation from cloacal swabs of SPF chickens inoculated at the 3rd day of life with 10^3 CFU of SE PT4.

It seems clear that bacteriophages need *Salmonella* in the alimentary tract to multiply. Negative results of bacteriophage isolations from birds of group 2 two days after inoculation indicates that no bacterium from the physiologic flora or other cell are targeted by these bacteriophages inside the alimentary tract of SPF birds. This observation characterizes the self-limiting nature of bacteriophages when inoculated in *Salmonella*-free birds.

All cecal contents of the birds from Group 1 were positive at necropsy for both *Salmonella* and bacteriophages. It was evident that *Salmonella* will not be cleared from the alimentary tract of SPF chickens by bacteriophages under the conditions used in the present experiment. However, reduction of CFU per gram of feces may occur and be useful in biocontrol of *Salmonella* in poultry. Persistence of SE PT4 in infected birds is common (Gast & Holt, 1998) and fecal shedding may persist for several weeks after infections (Shivaprasad *et al.*, 1990). No spleen or liver sample was positive for bacteriophage, even though they were *Salmonella* positive. Therefore, bacteriophages are unable to survive in internal tissues even if carried into the blood stream by bacteria, macrophages or other cells.

SE PT4 was isolated from spleen and ceca of all inoculated chickens. Cecal contents collected at necropsy showed a mean of $87.5 \pm 23.7 \times 10^8$ CFU per gram. This is an indication of heavy infection on inoculated chickens, even though a moderate infection dose (10^3 CFU/bird) was used (Asheg *et al.*, 2001). Isolation from spleen and ceca also indicates that SPF chickens were heavily infected by SE PT4, once this bacterium is likely to be cleared from internal tissues after two weeks when infection is performed with low doses (Asheg *et al.*, 2001; Kinde *et al.*, 2000). The high *Salmonella* contents in ceca may be a reason why *Salmonella* was isolated from every swab even though bacteriophages were present. Establishment of infection before the complete development of the physiologic microbiota and the maintenance of birds within filtered air cabinets are the likely reasons of this high infection rate even though the infecting dose was moderate.

All 35 isolates of *Salmonella* obtained from cecal contents at necropsy of birds from Group 1 remained sensitive to lysis by the three phages indicating that resistance to bacteriophages is not likely to develop *in vivo* within short periods. Also, none of these colonies showed the rough morphology that is indicative of selection under bacteriophage pressure. Recovery of bacteriophage-resistant SE after treatment of chickens with bacteriophages has been reported (Sklar & Joerger, 2001). Bacteriophage resistance may be related to specific viruses or experimental protocols.

CONCLUSIONS

The occurrence of bacteriophages lytic for SE PT4 and ST in free-range layers in Brazil can be demonstrated by culturing feces with susceptible *Salmonella*. The

bacteriophages that are more likely to be isolated present dsDNA as nucleic acids and show head and tail ultrastructure.

Results of this study suggest that bacteriophages CNPSA1, CNPSA3 and CNPSA4 do not remain in *Salmonella*-free birds longer than one day, although they multiply in *Salmonella*-infected birds for longer periods. Selection for phage-resistant SE PT4 did not seem to occur within short periods.

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