Research Note

Apparent Attachment of *Campylobacter* and *Salmonella* to Broiler Breeder Rooster Spermatozoa

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ABSTRACT It has been demonstrated that horizontal and vertical transmission of *Salmonella* and *Campylobacter* can occur in broiler breeder flocks. The mechanism of this transmission is still unclear. Previously negative broiler breeder flocks have been reported to become positive with *Salmonella*, *Campylobacter*, or both after the introduction of "spike" roosters at 45 wk of age. To determine whether the rooster semen is a possible source of transmission to hens for colonization, we evaluated the association of both *Salmonella* and *Campylobacter* spp. to segments (head, midpiece, and tail) of individual spermatozoa after artificial inoculation. *Salmonella typhimurium*, *Salmonella heidelberg*, and *Salmonella montevideo*, or *Campylobacter jejuni* (in 0.85% saline) was added to a freshly collected (by abdominal massage) aliquot of pooled semen from roosters housed in individual cages. The semen and bacteria solutions were incubated 1 h at room temperature. Samples were fixed using Karnosvsky and Zamboni fixatives for 24 h prior to centrifuging and rinsing in 0.1 *M* cacodylate·HCl buffer. Individual aliquot samples were then subjected to both scanning (JSM-5800) and transmission (JEM-1210) electron microscopy. The scanning electron microscopy showed that *Salmonella* was associated with all 3 segments (head, midpiece, and tail) of the spermatozoa and apparently equally distributed. *Campylobacter* was mainly associated with the midpiece and tail segments; few isolates were located on the head segment. The transmission electron microscopy showed apparent attachment of *Salmonella* and *Campylobacter* to the spermatozoa.

Key words: *Salmonella*, *Campylobacter*, spermatozoa, scanning electron microscopy, transmission electron microscopy

INTRODUCTION

Contamination of eggs by bacteria is of major concern to the poultry industry. This concern is not limited simply to the potential transmission of diseases to humans, but for passage of disease organisms to progeny. Contamination is generally thought to occur as the egg moves from the shell gland through the vagina and cloaca during oviposition (Smith, 1949; Reiber et al., 1991). Not only is there potential for contamination of the egg during oviposition, there is also potential for contamination of semen during copulation when semen is deposited by the male.

In chickens, the phallus is a small structure that arises when lymph fluid engorges the floor of the cloaca (Nishiyama, 1955). Semen is ejaculated from papilla at the terminus of the deferent ducts that are immediately lateral to the opening of the ureters (Sturkie, 2000). After release from the papilla, the semen flows down a centrally located longitudinal groove in the phallus. Birds lack the traditional accessory sex glands (i.e., prostate and bulbourethral glands or seminal vesicles) that are found in mammals (Lake, 1981). Sperm are suspended in seminiferous tubule fluid that is absorbed as the sperm cells transit the efferent ducts (Clulow and Jones, 1988). The composition and volume of this fluid are altered before entry into the epididymal duct, and the fluid is called seminal plasma thereafter (Esponda and Bedford, 1985). Chicken semen is concentrated with average ejaculate containing 3.5 billion spermatozoa/mL of semen (Lake, 1957). Turkey semen is more concentrated; values range from 6.2 to 7 billion spermatozoa/mL (McCartney and Brown, 1959).

During copulation, the phallus is placed against the everted cloaca of the female, and the ejaculated semen enters the lumen of the cloaca (Sturkie, 2000). The sperm must then traverse through the vagina and reach the primary sperm storage tubules in the vagina-shell gland junction. Chicken sperm have a very long, effective tail

that enables them to literally swim through the vagina to the sperm storage tubules. However, reverse-peristaltic action carries the spermatozoa the remaining length of the oviduct to the point of fertilization, the infundibulum (Lorenz and Ogasawara, 1968). Subsequently, the sperm reach the sperm secondary storage tubules in the infundibulum, where they may fertilize the recently ovulated ovum (Bakst et al., 1994).

Reiber et al. (1995) demonstrated that chicken semen is contaminated with a variety of bacteria. Furthermore, it has been demonstrated that both *Campylobacter* and *Salmonella* spp. have the potential to be vertically transmitted (Cox et al., 1990, 2002a, 2004). The mechanism of this transmission is still unclear. Previously *Campylobacter* negative broiler breeder flocks have been reported to become positive with the introduction of roosters at 45 wk of age through screening of fresh fecal droppings (Cox et al., 2002b, 2005). This colonization could be from mating or from oral ingestion of the feces.

Thus, it is possible that bacteria can attach to avian sperm, making them a potential source of horizontal and vertical transmission. The purpose of this study was to determine whether attachment of bacteria such as *Campylobacter* and *Salmonella* to the segments (head, midpiece, and tail) of individual spermatozoa of broiler breeder roosters could occur.

MATERIALS AND METHODS

Experimental Design

Broiler breeder roosters (n = 15) that were 46 wk of age were individually caged in a research setting and were trained for semen collection. The broiler breeder roosters were screened by fecal droppings for the presence of *Salmonella* and *Campylobacter* spp. for a 4-wk period prior to semen collection. *Salmonella* and *Campylobacter* spp. negative roosters (n = 6) were identified. Each rooster was abdominal-massaged, and the semen (approximately 1 mL) was aspirated into a 15-mL sterile polypropylene conical tube. The 6 semen samples were then pooled into 1 sample (5 mL), packed on ice, and transported back to the laboratory for inoculation and fixing.

Inoculum Preparation

For *Salmonella* preparation, pure *Salmonella* (*Salmonella typhimurium*, *Salmonella heidelberg*, and *Salmonella montevideo*) colonies were obtained from stock cultures from triptic soy agar slants. From each of the 3 different slants, the salmonellae were streaked onto brilliant green sulfur plates and incubated at 37°C for 24 h. *Salmonella* (*S. typhimurium*, *S. heidelberg*, and *S. montevideo*) were then picked from each of the 3 plates and placed into separate dilution blanks containing sterile water. Through use of a spectrophotometer, the inoculum level was determined and confirmed through appropriate dilutions.

For *Campylobacter* preparation, pure *Campylobacter jejuni* colonies were obtained from a bacterial preserver (treated beads in a cryopreservative fluid that are stored at –80°C). Three beads were placed onto *Campylobacter cefex* agar and incubated in a microaerophilic atmosphere at 42°C for 48 h. The *C. jejuni* colonies were then placed into 9-mL dilution blanks containing sterile water. Through use of a spectrophotometer, the inoculum level was determined and confirmed through appropriate dilutions.

Bacterial Inoculation of Semen

Extender (http://www.imvusa.com/) was added to the pooled semen samples at a 1:1 ratio. Two 1-mL aliquots from the pooled semen sample with extender were transferred to 12- × 75-mm sterile culture tubes. A 10^8 cfu/mL cocktail of *Salmonella* (*S. typhimurium*, *S. heidelberg*, and *S. montevideo*) was added to one of the semen/extender samples, and 10^6 cfu of *C. jejuni*/mL were added to the other semen/extender sample. The 2 inoculated solutions were then mixed and incubated for 1 h at room temperature and then fixed.

Fixative Process

Four samples were submitted in fixative for processing: 1) *Campylobacter* inoculum, 2) *Salmonella* inoculum, 3) spermatozoa inoculated with *C. jejuni*, and 4) spermatozoa inoculated with a cocktail of *Salmonella* (*S. typhimurium*, *S. heidelberg*, and *S. montevideo*). The fixative used is a modification of 2 electron microscopy fixatives, i.e., the Karnovsky fixative (Karnovsky, 1965) and the Zamboni fixative (Zamboni and DeMartine, 1967): 2% (para) formaldehyde, 2% glutaraldehyde, and 0.2% picric acid in 0.1 M cacodylate·HCl buffer (pH 7.25).

The samples were allowed to fix for 24 h prior to spinning down and rinsing several times in 0.1 M cacodylate·HCl buffer. Each of the 4 samples was then divided so that one-half of each sample was prepared for scanning electron microscopy (**SEM**), and one-half was prepared for transmission electron microscopy (**TEM**).

SEM

Glass coverslips were carefully cleaned with 95% ethanol. A drop of 1% Poly-L-lysine (Sigma Chemical Co., St. Louis, MO) was placed on each coverslip and allowed to dry for 1 h. The coverslips were rinsed in deionized water, and a drop of the fixed sample in buffer was placed on the coverslip. Two coverslips were used for each sample. The samples on coverslips were placed in a wet chamber and allowed to settle overnight (Mazia et al., 1975). Coverslips were taken from the wet chamber and rinsed in deionized water. The coverslips were passed through a graded series of ethanol (50, 75, 95, 95, 100, and 100% each for 10 to 15 min). The coverslips were loaded into an Autosamdri-814 Critical Point Dryer (http://www.tousimis.com/), and the samples were critical point-dried according to the manufacture's instructions. Once the samples were dried, each coverslip was

mounted onto an aluminum stub with Electrodag 502, a graphite adhesive (http://www.tedpella.com). After the adhesive had dried thoroughly, the stubs with the samples were placed in the SPI-Module Sputter Coater (http://www.2spi.com/), and 300 Å of Au was coated on the samples. The samples were then ready to be viewed with the JSM-5800 SEM.

TEM

The samples in fixative buffer were centrifuged, and the buffer was drawn off. The pelleted samples were enrobed in 3% molten noble agar at 58°C (http://www.difco.com/). Once the agar was cooled, the pelleted samples were cut out of their microfuge tubes and placed in buffer (Dawes, 1971). These samples were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate·HCl buffer. After rinsing in several changes of deionized water, pellets were placed in 0.5% aqueous uranyl acetate. After additional rinsing in several changes of deionized water, the samples were put through an ethanol dehydration series (50, 75, 95, 95, 100, and 100%). Dehydration was concluded with 2 changes in acetone, and the samples were cleared in 2 changes of propylene oxide. The samples were infiltrated with several changes of propylene oxide/Epon-Araldite (Mollenhauer, 1963) and then 100% Epon-Araldite before embedding in flat molds. The samples were allowed to polymerize overnight at 75°C.

The blocks containing the samples were trimmed to the location of the aggregation of cells using a Reichert Ultracut S Ultramicrotome (http://www.leica-microsystems.com/website/lms.nsf). Several 70-nm sections were cut and placed on 200-mesh Cu grids (http://www.ems-diasum.com/ems/). The grids were then poststained with 5% methanolic uranyl acetate and Reynold's lead citrate (Reynolds, 1963) before viewing with the JEM-1210 TEM.

RESULTS AND DISCUSSION

Electron microscopy has been used to study bacterial attachment to surfaces (Fletcher and Floodgate, 1973; Firstenberg-Eden et al., 1979; McMeekin et al., 1979), and this provided a method for rapidly determining whether *Campylobacter* or *Salmonella* could attach to avian spermatozoa. Through use of electron microscopy (both SEM and TEM), *Salmonella* (Figure 1) and *C. jejuni* (Figure 5) were found to be apparently attached to the broiler breeder rooster spermatozoa (Figures 2, 3, 4, 6, 7, and 8). In general, when evaluating the numerous fields utilizing SEM for both *Salmonella*-spiked semen and *Campylobacter*-spiked semen, *Salmonella* tended to have a higher association with spermatozoa than did *Campylobacter*. However, this observation could have been due to the lower inoculum level of *Campylobacter* than *Salmonella*. The lower inoculum level would give a higher spermatozoa:bacteria ratio per milliliter. Furthermore, >1 cell (either *Salmonella* or *Campylobacter* cells) was observed numerous times apparently attached to spermatozoa. In some instances, as
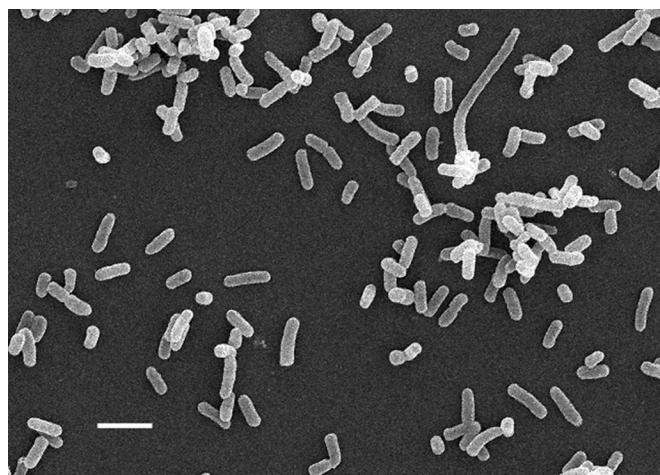

Figure 1. Scanning electron micrograph of the *Salmonella* used in the inoculation of the broiler breeder rooster sperm (bar = 2 μm).

many as 6 to 8 bacterial cells of *Salmonella* were apparently attached to a spermatozoon, where *Campylobacter* cells were usually only 1 to 3 per spermatozoon.

Salmonella were found associated and equally distributed to all 3 segments (head, midpiece, and tail) of the spermatozoa. In Figure 2, the cell membrane of a *Salmonella* cell is apparently extending to the head and midpiece region of a spermatozoon. In Figure 3, three *Salmonella* cells can be seen apparently attached to the head and midpiece region of a spermatozoon, and in the background, 1 *Salmonella* cell is apparently attached to the tail section of another spermatozoon. In Figure 4, through use of TEM, the cell membrane can be seen further extending to the head region of a spermatozoon. Using bull and boar sperm, Ahluwalia et al. (1990) found that the plasma membrane of the sperm head contained many oligosaccharides, and the midpiece and tail contained a moderate amount of oligosaccharides. Oligiosaccharides
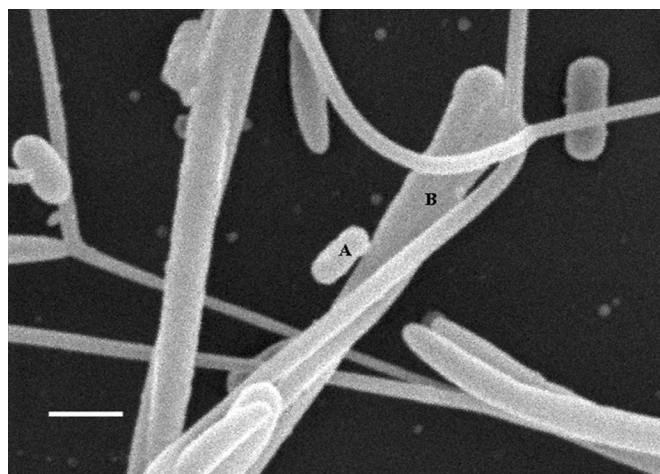

Figure 2. Scanning electron micrograph of the apparent attachment of *Salmonella* to a broiler breeder rooster spermatozoa (bar = 1 (μm). A = Apparent attachment and projection of the *Salmonella* cell membrane to the broiler breeder rooster spermatozoon; B = head and midpiece regions of spermatozoon.
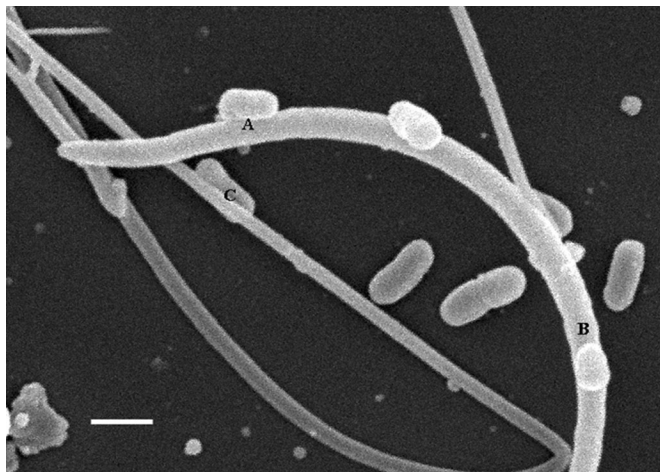
Figure 3. Scanning electron micrograph of the apparent attachment of 3 *Salmonella* cells to the head and midpiece region of a spermatozoon and apparent attachment of 1 *Salmonella* cell to another spermatozoon tail region (bar = 1 μm). A = Apparent attachment of a *Salmonella* cell to the head region of a spermatozoon; B = apparent attachment of a *Salmonella* cell to the midpiece region of a spermatozoon; C = apparent attachment of 1 *Salmonella* cell to another spermatozoon tail region.

have been demonstrated to prevent attachment of *Salmonella* to the intestinal epithelium (Sharon et al., 1981). This would suggest that *Salmonella* attachment to the sperm would be on the tail and midpiece. However, in the present study, *Salmonella* were found associated with all 3 segments (head, midpiece, and tail) of the spermatozoa (Figures 2 and 3).

Campylobacter jejuni was mainly associated with the midpiece and tail segments; few isolates were located on the head segment. In Figure 6, *C. jejuni* is coiled around the tail section of one spermatozoon. In Figure 7, *C. jejuni* appears to be coiled around the head of a spermatozoon,
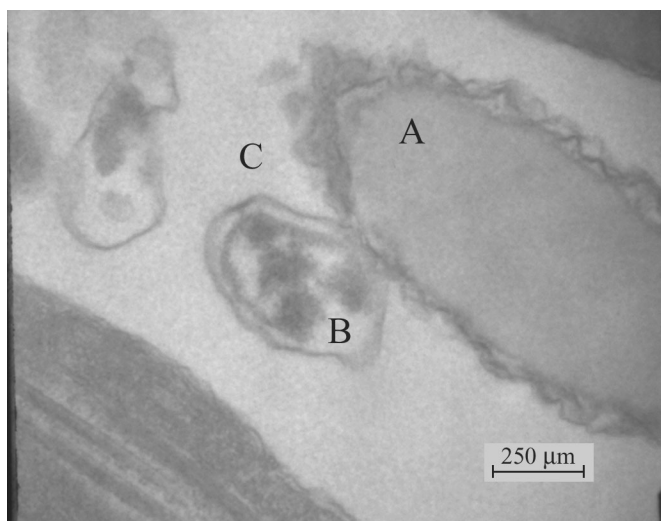

Figure 5. Scanning electron micrograph of the *Campylobacter jejuni* used in the inoculation of the broiler breeder rooster sperm (bar = 1 μm).

and cell membrane interaction is occurring. Also in the current study, *C. jejuni* was mainly associated with the midpiece and tail segments; few isolates were located on the head segment (Figures 6 and 7). *Campylobacter* spp. are reported to have an affinity for unsaturated fatty acids (Szymanski and Armstrong, 1996). About 50% of the fatty acids in the avian sperm plasma membrane are polyunsaturated (Surai et al., 1998). In addition, the phospholipid phosphatidylcholine is abundant in the head and midpiece of sea urchin sperm, and most of the fatty acid moieties in the sea urchin midpiece are unsaturated (Mita et al., 1991). Perhaps *Campylobacter* species bind specifically to the polyunsaturated fatty acids of the avian spermatozoa plasma membrane.

Both *Salmonella* and *Campylobacter* could be vertically transmitted (Cox et al., 1990, 2002a; Cox and Bailey, 1991). However, the mechanism of this transmission is unclear. The anatomy of the reproductive tract of both male and female chickens is such that semen is exposed to the

Figure 4. Transmission electron micrograph of the apparent connection of the cell membrane of a *Salmonella* cell and the membrane at the head region of a broiler breeder rooster spermatozoon (bar = 250 μm). A = Head region of spermatozoon; B = *Salmonella* cell; C = apparent connection of the cell membrane of the *Salmonella* cell to the spermatozoon.
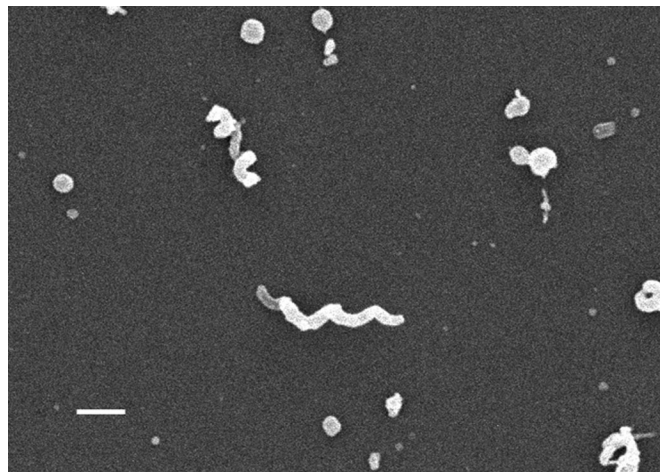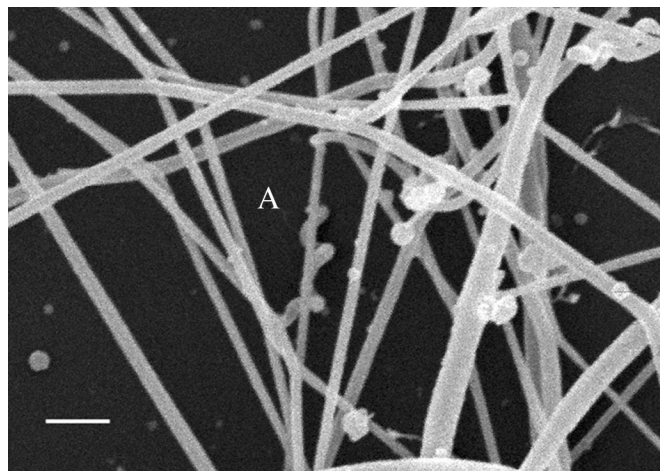

Figure 6. Scanning electron micrograph of *Campylobacter jejuni* coiled around the tail section of one spermatozoon (bar = 1 μm). A = *Campylobacter jejuni* coiled around the tail section of a spermatozoon.
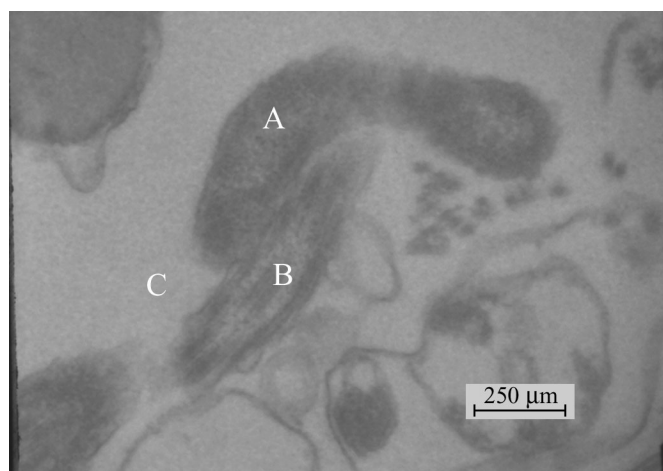
Figure 7. Transmission electron micrograph of *Campylobacter jejuni* apparently connected and coiled around the head region of a broiler breeder rooster spermatozoon (bar = 250 μm). A = *Campylobacter jejuni*; B = head section of a spermatozoon; C = apparent connection of the cell membrane of *C. jejuni* to the spermatozoon.

cloacal contents of both sexes, providing ample opportunity for bacterial contamination. Thus, the opportunity for spermatozoa providing transportation for bacteria to the developing ova exists. Transport of bacteria by spermatozoa has been considered in human sexually transmitted diseases such as gonorrhea. In fact, both pileated and nonpileated *Neissera gonorrhoea* have been shown to attach to human sperm (James-Holmquest et al., 1974). However, it is important to remember that this study was conducted with spiked semen samples to increase the bacteria:spermatozoa ratio. The average sperm of roosters contains approximately 3.5 billion spermatozoa/mL. Therefore, after the addition of extender, the semen had approximately 1.7 billion cells/mL. With an inoculum of 10^8 cfu/mL for *Salmonella* and an inoculum of 10^6 cfu/mL for *Campylobacter*, this would give a greater bacteria:spermatozoa ratio than you would find with natural semen samples. Therefore, the association of these bacteria to the different sections of a spermatozoon could be different in naturally contaminated semen.

In this study, it was determined that apparent attachment of both *Campylobacter* and *Salmonella* to broiler breeder rooster spermatozoa could be possible, and this could ultimately be taxis for these bacteria to enter other host or progeny. However, further research needs to be conducted to conclusively determine whether attachment to spermatozoa is occurring. If this is true attachment of bacteria to the spermatozoon, then research needs to be conducted to determine the methods bacteria utilize to attach to broiler breeder spermatozoa because spermatozoa are not known to have receptor sites for bacteria. In addition, the stability of the attachment of bacteria to the spermatozoa would need to be determined because of the travel of the spermatozoa up the female reproductive tract and also the penetration into the ovarian follicle of a hen. Nevertheless, this study provides evidence that *Campylobacter* and *Salmonella* could have the ability to attach to broiler breeder rooster spermatozoa, and this could be a mode of transmission into broiler and breeder flocks.

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