Biofilm building properties of salmonella on the poultry farm

by Dr Esther Schonewille, Dr Daniel Windhorst and Dr Ina Bräunig, Lohmann Animal Health GmbH, Heinz-Lohmann-Straße 4, 27472 Cuxhaven, Germany.

Biofilms in nature usually persist attached to some surface and not as pure cultures of unattached or so-called planctonic growth. Varying definitions exist but biofilm is frequently described as an assembly of microbes attached to a surface and being embedded in a matrix of extracellular polymeric substances.

Potential salmonella haven

A very important aspect of biofilms in primary poultry production is that they can be a potential harbour for salmonellae as well as Campylobacter jejuni in the field and are often mentioned in respect to enhancing the resistance and virulence of salmonella.

It was demonstrated that in Norwegian fish feed factory settings certain salmonella strains persist for years and are capable biofilm producers. Interestingly these salmonella connected to biofilm have been proven to originate from a few limited clones from these settings.

Contrary to persisting isolates, isolates with limited property to form biofilm were more often isolated from the fish feed factory setting and as the crucial difference showed a remarkably more diverse clonal variety. We wanted to understand whether the same observation could also be made for farm-isolated salmonella serotypes with high importance for food safety.

Do especially hard to eradicate serotypes such as S. paratyphi B d-tartrate positive warrant an in-depth study of their biofilm forming properties? Can a link be found between the serotypes found in chicken production, their biofilm building property and their clonal variety?

Hence, we have taken a variety of strains from various serotypes of Salmonella enterica with major impact on the poultry industry in Germany and Hungary and examined these for their biofilm-building properties in a laboratory setting.

To understand the common origin of the selected strains the results were linked with epidemiological data, such as pulse field gel electrophoresis (PFGE) and lysotyping.

Material and methods

Altogether 78 different salmonella field strains belonging to eight different serotypes were examined as well as eight laboratory adapted strains. Six live vaccine strains and vaccine precursor strains were also included in the analysis.

Biofilm-building property can be judged by employing a simple laboratory assay that involves transferring the strains to a stationary 96 well plate and measuring the amount of biofilm formed over a 48 hour period. We looked at traits such as biofilm-building property at 20°C and at 37°C as well as epidemiological relatedness.

The classical approach of pulse field gel electrophoresis (PFGE) was employed to understand the relatedness of investigated salmonellae as well as the more specialised lysotyping for S. infantis strains from Germany and Hungary.

Results

- **Different serotypes:**

Not surprisingly the property to build biofilm is strongly dependent on the serovar. In general the infamous Salmonella enteritidis is a strong biofilm producer, followed by S. paratyphi B, d-tartrate positive, S. livingstone, S. virchow, S. saintpaul and S. infantis. S. gallinarum did not produce any detectable biofilm in our laboratory set-up. We can only speculate that this is due to the absence of fimbriae in S. gallinarum as we have not tested enough different S. gallinarum strains to verify this assumption (Fig. 1).

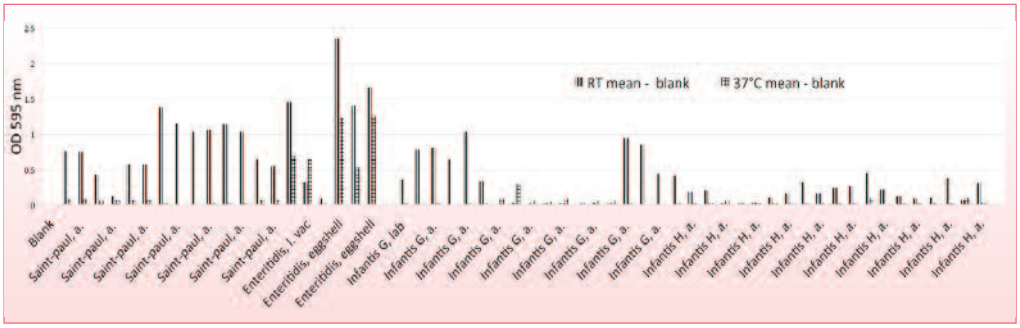
- **Genetic and phenotypic relation:**

It does not stop here; considerable differences could also be observed within a certain serovar. For example S. enteritidis isolated from eggshells are very apt at producing biofilm, whereas S. enteritidis that have been passaged in rich media over decades in the laboratory are very poor biofilm producers. The same holds true for different strains from the serovar S. paratyphi d-tartrate positive (formerly known as S. java), where strains coming from the farm environment are good biofilm producers and laboratory adapted strains are poor biofilm producers (Fig. 2). Norwegian investigations for the feed mill factory environment have shown that strains with similar biofilm-building properties when coming from similar environments were closely related genotypically. For example, all tested S. paratyphi B, d-tartrate positive strains clustered within 94% similarity in PFGE analyses.

- **Temperature:**

Under the reported conditions biofilm-building property is also strongly dependent on the temperature used for incubation (Fig. 1). Different S. saintpaul isolates from a farm environment incubated at 37°C showed a mean biofilm-building property as expressed via measured OD_{595} levels of 0.0645. The same isolates showed a significantly higher and pronounced biofilm building property resulting in a mean OD_{595} of 0.75 when they were incubated at 20°C. Similar observations could be made for isolates of S. paratyphi B, d-tartrate positive and isolates of S. entertidis. S. infantis isolates however were poor biofilm producers in general, independent of the incubation temperature. A total of 26 strains obtained from Hungarian broiler farms showed a biofilm-building property below the cut-off value of OD_{595} 0.5, whereas three isolates from one German farm that clustered together in PFGE analysis (98%) (results not shown) all produced biofilm well above the set cut-off.

Fig. 1. Biofilm building property of salmonella isolates from different serovars at 20°C and at 37°C, a = isolate of animal origin; lab = laboratory adapted strains; l. vac. = strain for live vaccination; G = German isolate; H = Hungarian isolate.



Discussion

Our initial question was whether salmonella isolates important to poultry production and with major impact on food safety are involved in the formation of biofilm in a laboratory setting. Are there differences in

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biofilm production between or within different salmonella serovars? Do these differences reflect different epidemiological backgrounds?

Limited knowledge on biofilm-producing salmonellae is available in poultry production. We clearly demonstrated that the incubation temperature has a very strong influence on biofilm-building properties.

At 20°C considerably more biofilm is produced than at 37°C. We interpret this as a possible reflection of less favourable environmental conditions for salmonella at room temperature (RT) and hence less need to produce protective biofilm.

Although this simple explanation might sound reasonable it has to be noted that in contrast to these observations Spanish studies from the 1990s reported that 71% of the investigated S. enteritidis isolates of human and river water origin were able to build biofilm at 20°C and 37°C.

The assay was based on pellicle formation at the liquid-air interface in rich and reduced media.

Interestingly it was observed that biofilm-building property was stronger at higher temperatures in human as well as river water isolates.

No laboratory-adapted salmonella isolates or any vaccine precursor and live-vaccine strain isolates were included in their investigation. Finally they reached the conclusion that the composition and regulation of biofilm depended to a large extent on environmental conditions.

Salmonella typhiumurium

For S. typhimurium it is described that strains from the same salmonella phage type have variable survival profiles on the same surfaces. Following this observation for S. enteritidis we were able to show that strains recovered from eggshell surface had a significantly higher biofilm-forming property as did strains that had been kept under laboratory conditions.

S. enteritidis strains can contaminate eggshells and the property to form a biofilm certainly is a helpful phenotypic trait for such strains.

Strains that are able to invade the reproductive tissue of laying hens and henceforth contaminate the outer egg shell membrane also display increased tolerance to environmental conditions such as heat, acid, and H_2O_2. These traits are also characteristic for strains isolated from an environment favouring the development of biofilm.

Furthermore, two of our S. paratyphi B, d-tartrate positive isolates were recovered over a time period of 18 months originating from one clone, as demonstrated by 98% similarity in PFGE. Both isolates were able to produce copious amounts of biofilm on both isolation dates.

As recently described for the fish and feed mill environment in several Norwegian studies this has considerable consequences with regard to persistence over several production cycles in primary poultry production.

For us it was interesting to observe that S. infantis is overall a rather poor biofilm producer as it is the serovar with the highest prevalence in Hungary. All 26 isolates obtained from Hungarian broiler farms showed biofilm below the cut-off value of OD 0.5.

However isolates from three different German farms that cluster together in PFGE analysis (98%) all produced biofilm above the defined cut-off value. It can be hypothesised that the persistence of S. infantis on Hungarian broiler farms is not due to their biofilm-forming properties.

This leads to the assumption that the high S. infantis presence in Hungary could be a result of its widespread distribution in the Hungarian broiler sector as opposed to the persistence in several sturdy clones, as might be the situation in Germany, where S. infantis prevalence is markedly lower.

To strengthen this particular argument the variable epidemiological picture of S. infantis in Hungary is useful. The 26 Hungarian strains

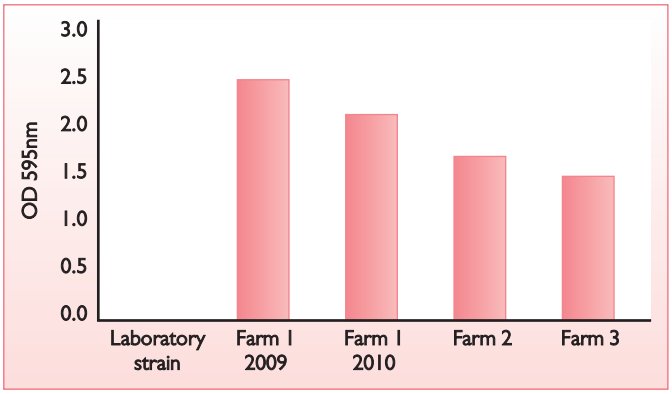

Fig. 2. Biofilm building properties of S. paratyphi B, d-tartrate positive isolated from poultry farm settings as compared to a laboratory adapted strain.

cluster in two large PFGE clusters (74%) spaced far apart and represent five different lysotypes (LT).

Controlling biofilm

Numerous techniques exist that promise to control biofilm build-up in different settings. Effective means can be for example mechanical instruments, such as pulsed air-pressure cleaning devices. These have already demonstrated their usefulness and effective cleaning properties under field conditions.

Surface acoustic waves are being successfully used in human urinary catheter care but have not been tested in practical settings in primary poultry production at all.

Antimicrobial coated components are also quoted as helpful in reducing biofilm build-up in primary poultry production.

Several substances exist that exhibit an acceptable amount of effectiveness in suspension tests, such as hypochlorite, ethanol (70-80%), tensides, and oxidative disinfectants. However, surface tests are more relevant under practical conditions when it comes to judging possible effectiveness of substance against biofilm. Hence when using the classical substance approach in biofilm combat, proper use of the substance in question is of paramount importance. Does the substance have proven efficiency in the proper concentration and under practical conditions (temperature, pH, and organic content)?

A different way of using chemical approaches to control biofilm formation is by disrupting the recently heavily-discussed mechanisms of quorum sensing. The way bacterial communities communicate amongst each other may be interfered with through the use of nucleotide synthesis inhibitors, such as furanones.

Experimental quorum sensing inhibitors have been successfully applied to the inhibition of biofilm formation in Staphylococcus aureus and Pseudomonas aeruginosa.

Conclusion

Despite all these listed possibilities of combat it is important to understand that biofilm-building property is a function of adaptation to the host environment. Since biofilm can also form a habitat for salmonella in farm environments and not only in laboratory settings, its control is of paramount importance to the overall improvement of food safety.

Special attention must be paid to environments which are notoriously difficult to decontaminate, for example feed mills and primary poultry production. ■