Numeric taxonomy approaches for lytic evaluation of *Salmonella* specific bacteriophages

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A B S T R A C T

This study explored the lytic ability of bacteriophages as a future tool for reducing the *Salmonella* spp. loads in food animals. It investigated (a) the concept of a phage cocktail resulting from an exploratory analysis of the 13 phages which were examined, and (b) the possibility of using them in phage typing techniques for a broad range of serotypes. By using the conventional plaque assay method and cluster analysis, it was possible to select the 2/2, N5, 2α, 135KP and 12′ phages, as potential elements of a cocktail as a means of efficiently eliminating the greatest number of several types of *Salmonella*. The 2/2 and N5 phages were also the most efficacious infective elements against the Typhimurium and Enteritidis serovars, respectively.

Keywords:
Salmonella spp.
Bacteriophages
Spot test
Lytic spectra
Phage typing
Cluster analysis
Discriminant analysis

1. Introduction

Outbreaks of food-borne disease with identified aetiology have been predominantly of bacterial origin, primarily *Campylobacter* and *Salmonella* (Cardinale et al., 2005). Animal husbandry practices used in the poultry, meat and fish industries, and the recycling of offal and inedible raw materials into animal feeds, has favoured the continued prominence of *Salmonella* in the global food chain (Beuchat, 1998). Although most infections cause mild to moderate self-limited disease, serious infections leading to death do occur. Each year in the United States of America for example, food-borne diseases result in an estimated 76 million illnesses, 325000 hospitalizations and 5000 deaths (WHO, 2007).

Evaluating the lytic ability of bacteriophages to infect a particular type of bacteria may allow the use of these agents to eliminate, control or identify those cells in food (Greer, 2005). Phage-based control of pathogens has been demonstrated for some bacterial species on certain food products such as tomatoes (Balogh et al., 2003), sprouts (Pao, Randolph, Westbrook, & Shen, 2004), chickens (Huff et al., 2002; Smith et al., 2000) and beef cattle (Bach, McAllister, Vieira, Gannon, & Holley, 2002), namely to reduce *Salmonella* loads (Higgins, Higgins, Bielke, & Hargis, 2007; Higgins et al., 2005; Pao et al., 2004). The results of these studies are promising, with significant reductions of pathogenic bacteria observed. The application of bacteriophages to bacterial infections of humans or other animals with the goal of reducing bacterial loads is so-called phage therapy. For that, free phages can be used as the usual method of delivery to infections, or unusually, phage-infected bacteria may be employed. Phages also may be genetically engineered to deliver known phage genes coding for antibacterial agents (Goodridge & Abedon, 2003; Westwater et al., 2003).

The host specificity of bacteriophage is such that it is possible to differentiate strains or even within serotypes (as for the case of *Salmonella*) (Sklar & Joerger, 2001) of individual species of bacteria on the basis of their susceptibility to various kinds of bacteriophage – phage typing (Brown, 2005). Investigating with this procedure makes possible the determination of the host range of a particular phage, enabling a selection of broad specificity of phages for most salmonellae and to differentiate strains using some of the tested phages. Phage typing has been used as an identification tool for effective bacterial diagnosis. Various phage-based bacterial diagnostic techniques have been described. Bacteria may be identified by their release of virion particles upon cell lysis. These "phage amplification assays" have been applied for the rapid detection and identification of specific pathogenic bacteria including *Salmonella* and *Escherichia coli* O157:H7 (Favrin, Jassim, & Griffiths, 2003).

Similarities may be established and performed by cluster analysis for a better understanding of results to provide the definition

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of clusters, identification schemes and the selection of representative strains for allied studies (Sneath & Sokal, 1963). In spite of being old methods of exploratory analysis, recent studies have applied them in microbiology research. Hierarchical, cluster analysis method, UPGMA (sequential agglomerative hierarchical non-overlapping) was used to establish the genetic distance among microbe strains (Abaidoo, Keyser, Singleton, & Borthakur, 2002; Blackall, Fegan, Chew, & Hampson, 1998; Koeuth, Versalovic, & Lupski, 1995; Mahmoud, Gaafar, & Mubarak, 2007; Ochiai, Inoue, Takeya, Sasaki, & Kaku, 2005; Smith et al., 2000). Non-hierarchical, ordination analysis methods, principal component analysis (PCA) and principal coordinate analysis (PCO) are used for understanding the taxonomic structure, giving a realistic representation of distance between major groups; they work as a means of confirming whether suggested hierarchical groupings are really valid (Sneath & Sokal, 1973). These methods were used to find metabolic, genetic and epidemiological relatedness among microorganisms in several studies (Chapman et al., 2006; Feldgarden, Byrd, & Cohan, 2003; Park et al., 2006; Smith et al., 2000).

The aims of this study were to select a group of phages with broad specificity for most salmonellae, but particularly serovars Enteritidis and Typhimurium, and to differentiate strains of *Salmonella* using some of the tested phages.

2. Materials and methods

2.1. Bacteriophages

Thirteen different phages tested in this study (ϕ12, ϕ31m, ϕ39, ϕ68, ϕ135KP, ϕ135m, ϕ169, ϕ2/2, ϕ2/2*, ϕN5, ϕ38, ϕ2α, ϕ2β) had been isolated, characterized and are held in the EC Project Phagevet-P culture collection by University of Minho, Braga. An archival stock of each phage strain was maintained in a solution of 0.90% NaCl (w/v) under refrigerated storage. To enumerate the phages in these experiments, the plaque assay method, preceded by serial decimal dilutions in 0.90% NaCl (w/v), was performed (Chapman et al., 2006). Archival stocks of phage suspensions contained ca. 10^{10} plaque forming units/ml (PFU/ml).

2.2. Bacterial strains

Ten bacterial hosts were taken and checked for the absence of prophage in a previous study done by University of Minho and NII-Genet (National Institute for Immunology and Genetics, Moscow). In addition to these 10 *Salmonella* strains, 67 more were arbitrarily selected from the ESB *Salmonella* culture collection. Most of the collection strains came from clinical salmonellosis cases that had occurred in several hospitals of the regions of Porto, Braga and Guimarães. Others were isolated from food, namely from smoked sausage, Frankfurter, pork, chicken or turkey meat in the North of Portugal. Most of the tested isolates belong to the Enteritidis serovar (45%), followed by the Typhimurium isolates (21%). Archival bacterial cultures were conveniently maintained at −70 °C with a cryoprotectant (30% v/v glycerol). During the experimental period, all the isolated cultures were stored, in the laboratory of analysis, at −20 °C, in TSB containing 30% glycerol (v/v).

2.3. Phage infection

Colonies were sub-cultured from Tryptic soy agar (TSA; Pronadisa, 37 °C, 18 h) into fresh tryptic soy broth (TSB; Pronadisa) and grown overnight, at 37 °C. An inoculum of 0.1% v/v of these cultures was transferred to fresh TSB on the day before the experiment to ensure that all cells were in the same growth phase at the time of infection (18 h, 37 °C). The cells were harvested by centrifugation at 163×*g* for 5 min. The pellet was re-suspended in saline solution (0.90% w/v NaCl). Aliquots (150 μl) of a 10^8 CFU/ml bacterium inoculum were placed in sterile tubes in duplicate, and 5 ml of molten cooled (44 °C) TSA – "top agar" (0.5% w/v agar) was added. The mixture was homogenized and poured into plates. After solidification, aliquots (5 μl) of each phage suspension (containing ca. 10^9 PFU/ml in 0.9% w/v NaCl) were placed on the inoculated 'top agar' surface ('spot test'). During the infection assays, concentrations of bacteria were 10^8 CFU/ml (calculated from colony) counts on TSA plates incubated overnight at 37 °C, using the Miles-Misra method (Miles, Misra, & Irwin, 1938). The tested phages were produced three times and tested (in duplicate) in eight experiments against all 77 *Salmonella* isolates, to assess reproducibility. In this study a single soft agar layer was used without any added cations (Mg^{2+} or Ca^{2+}) to benefit the phage infection, creating the minimum conditions for infection. This was a precedent study for application of bacteriophage suspensions in food or food animals, and often this environment does not offer such optimised conditions.

2.4. Data analysis

To analyse these results, two kinds of approaches have been used: (a) presence/absence of visible plaque formation (halo on susceptible strain) – binary analysis; (b) measurement of the halo (plaque) diameter (in millimetres) – quantitative analysis. For this study, if at least five assays of each variable pair (phage-*Salmonella* isolate) were positive, the set of experiments was classified as positive. When less than five positives were observed, the isolated bacterium at issue was classified as resistant (and the phage, non-infective). Calculations were performed and information was analysed by inserting the data in both NTSYSpc 2.0 (NTSYSpc 2.20d, (C) 1986–2005, Applied Biostatistics Inc.) program for cluster analysis, and SPSS 13.0 for discriminant analysis.

The phage lytic spectra analysis allowed an analysis of the phage global performance. These data were coded to a binary type of characters; one for all positives, or zero for all negatives. The estimation of resemblance between the elements was made by using the simple matching coefficient. Descriptions of the coefficients are given in many texts (Legendre & Legendre, 1983; Pielou, 1984; Sneath, 1963). The proportionality and independence be-

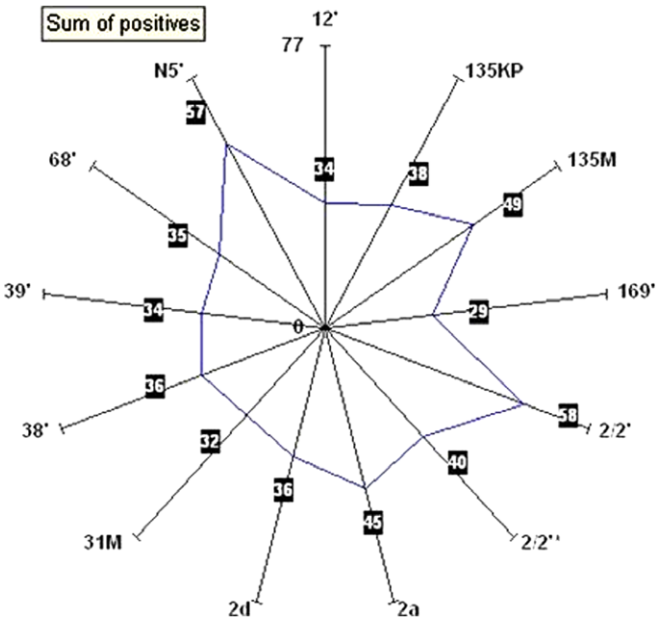

Fig. 1. Phage global performance.

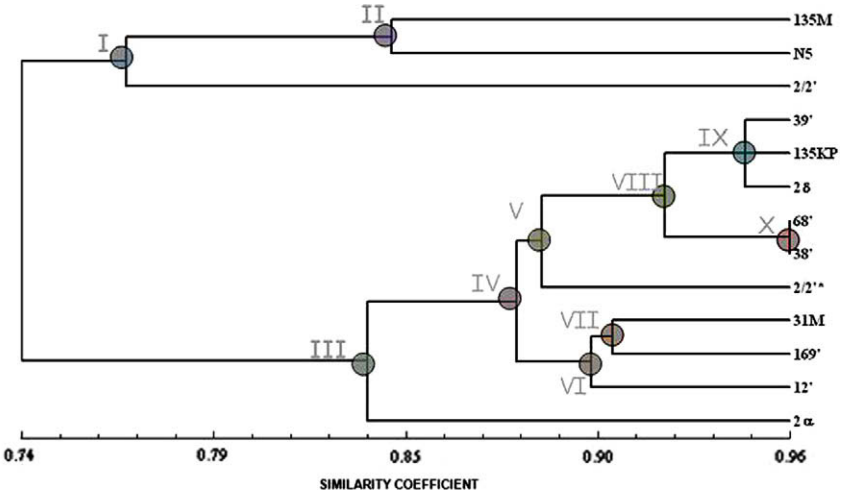

Fig. 2. Dendrogram establishing the relationship between the studied phages (SAHN/UPGMA).

tween pairs of operational taxonomic units (OTU's) vectors was established with the agglomerative sequential, agglomerative, hierarchical, and nested (SAHN) analysis (Dunn & Everitt, 1982; Sneath, 1973), by the clustering unweighted pair-group method arithmetic average (default) (UPGMA) method. The distances between OTU's were minimized by using the principal coordinate analysis (PCO).

The evaluation of phage fitness, regarding the plaque size, was preceded by the average and standard deviation calculation for each phage-isolate pair. The values considered for the average calculation were the ones in which the set of experiments was classified as positive. Standardization of data was made to return a normalized value from a distribution characterized by mean and standard deviation: $x' = (x - X)/\mathrm{SD}$, where x is the value to normalize, X is the arithmetic mean of the distribution; SD is the standard deviation of the distribution. The data obtained were elaborated with the simple matching similarity coefficient, applying the SAHN clustering method. Using the principal component analysis (PCA), a correlation matrix of characters was built, representing the OTUs in the new system of axis and reduced dimensionality to retain a significant portion of the initial variance.

A cluster descriptive analysis has been performed for quantitative data. The aim of this application is to build a "model" of how it can best predict to which group a case belongs (Park et al., 2006). The Kolmogorov–Smirnov (K–S) was performed to check if the variable distribution is normal (Dallal & Wilkinson, 1986).

3. Results

3.1. Phage lytic spectra

Fig. 1 displays axes representing each phage and the corresponding susceptible units (bacterial isolates) perceptual value. Both phage 2/2 and N5 are the ones that lyse the majority of the tested isolates (58 and 57 of 77, respectively). The phages 135M and 2α also seem to have large lytic spectra. The phage 169′ is the one that lyses the least; only 29 of 77 isolates were susceptible to infection by this phage.

The dendrogram (Fig. 2) allows one to establish a relationship between the phages, in view of their performance in lysing *Salmonella* isolates. Thus, the phages that infect the same isolates present an equal coefficient of correlation, as observed for the phages 68′ and 38′, meaning that they have a more similar behaviour in the test infection. A phage set with similar performance is the group IX (phages 39′, 135KP and 2δ), being the second more similar group. The phages from the group I do have an action profile far-away from the others (related to group III). This dendrogram coefficient of cophenetic correlation has been determined as 0.89, corresponding to a good fit ($0.8 < \mathrm{ccc} < 0.9$) (Sneath, 1963).

The principal coordinate analysis was performed in order to minimize distances and re-examine the distribution of phages (Fig. 3). The phages 2/2′, N5 and 135M are quite distant from the rest of the elements. The phages 38′, 68′, 39′ and 2δ seem to have the same behaviour in the test infection, but notice that the overlap between them in the plot is based on the first two dimensions only. Indeed it is possible to separate two major groups from which two more efficient infective phages can be selected; phage 12′ and 135KP. Few other phages are projected sparsely in the space, indicating that their performance in lysing the tested *Salmonella* isolates can be distinguished by the number and the type of susceptible strains.

3.2. Evaluation of phage fitness, regarding the plaque size

A dendrogram of distances (ccc = 0.875) was elaborated and almost all the phages were shown to be distinguishable with a few cases of probable homonymy (Fig. 4). The phages 2/2, N5 form an independent branch, being the more distinct elements of the global OTUs. The phages 38 and 68 (κ group) form the most similar group. The second more related group is the one with the phages 135KP, 2 (ι group). The PCA (significant portion of initial variance = 54.91%) graph (Fig. 5) shows that the assays which present larger halos occur with the phages disposed on the right side from the centre of the graph. There are also phages whose efficiency is notable by the lower number of non-susceptible isolates. The two marked groups include elements with very similar performance, suggesting the selection of the strongest virus (lysing the largest number of salmonellae and with the largest halos).

The stepwise discriminant analysis showed that the percentage of correctly classified elements is 92.3%. From the estimation of resemblance between the bacterial isolates, a dendrogram could be constructed describing a system of nested clusters (Fig. 6). The "serovars" characterization was performed and highlighted by the use of different colours for each one. The blue[1] branches correspond to the isolates of *Salmonella enteritidis*; in red are the iso-

[1] For interpretation of color in Figs. 1–6, the reader is referred to the web version of this article.

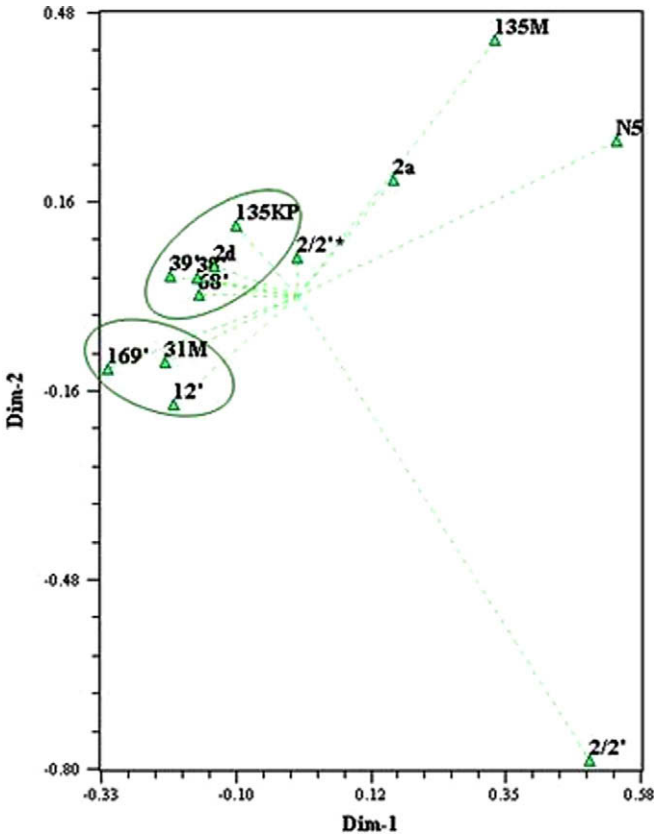

Fig. 3. Principal coordinate analysis of 13 phages.

lates of *Salmonella typhimurium*; the remaining groups adopt different colours; and, in black are the ones whose serovars are unknown. The isolates from the Enteritidis group are in two main branches (ν and λ groups). The ν group includes, mainly, food source isolates and the λ group includes only clinical isolates with the exception of the isolate 71. Of the Enteritidis isolates, 97.1% are located in one of the two mentioned groups. The ν group derives from the same node as the branch of the Typhimurium isolates,

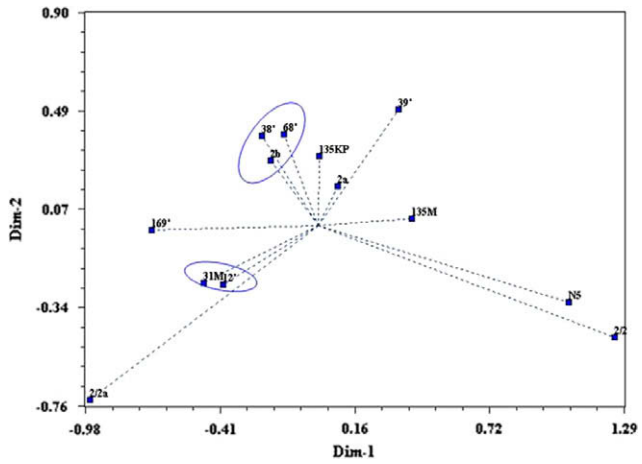

Fig. 5. Ordination plot of phages (PCA).

together with the isolates from other less representative serovars (*S.* Infantis, *S.* Rissen, *S.* Derby, *S.* Bredeney, *S.* Goldcoast, *S.* Virchow, *S.* GII, O4,12:z:1) (θ group). Of the Typhimurium isolates, 87.5% are found within this group.

4. Discussion

In spite of some researchers considering that measuring the plaque diameter is not a valid analysis for evaluating the efficacy of phage infection, many others think that determining the phage fitness during plaque growth is extremely important since different plaque regions may be under different selective pressures that can vary over the course of plaque development (Lee & Yin, 1996; Wilson & Miles, 1946). Nevertheless, plaque size does not necessarily correlate with per-infection productivity (burst size). It has been hypothesized, for instance, that phages displaying shorter latent periods, even given smaller burst sizes, could display larger plaques (Koch, 1964; Yin & McCaskill, 1992).

Using the classical method of detecting the infection, production of plaques on a lawn, the most infective phages are 2/2 and

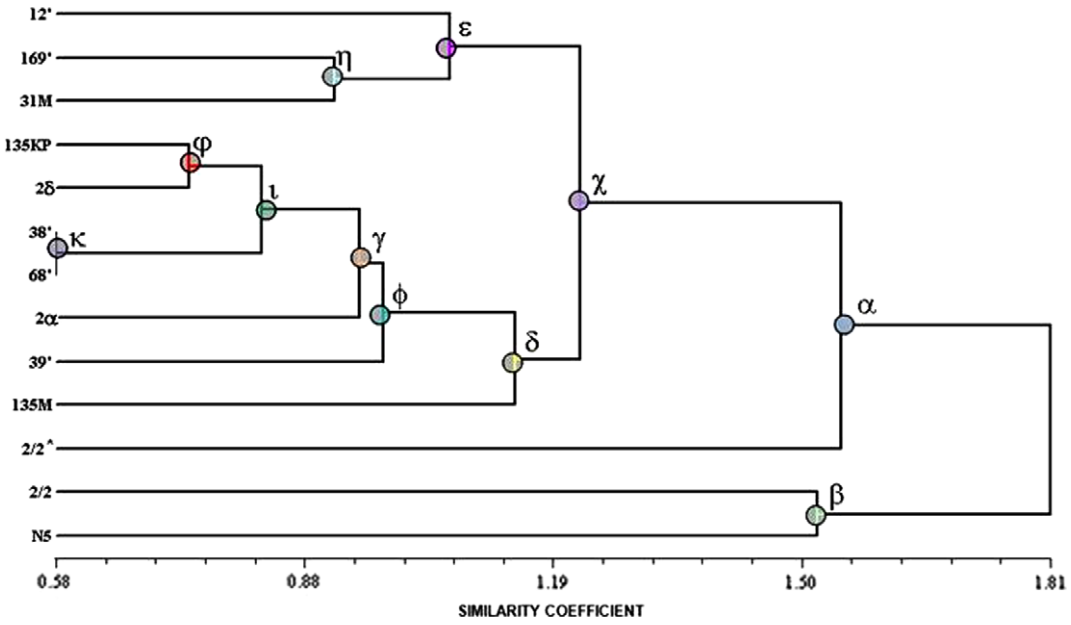

Fig. 4. Dendrogram establishing the relations between phages (SAHN/UPGMA).

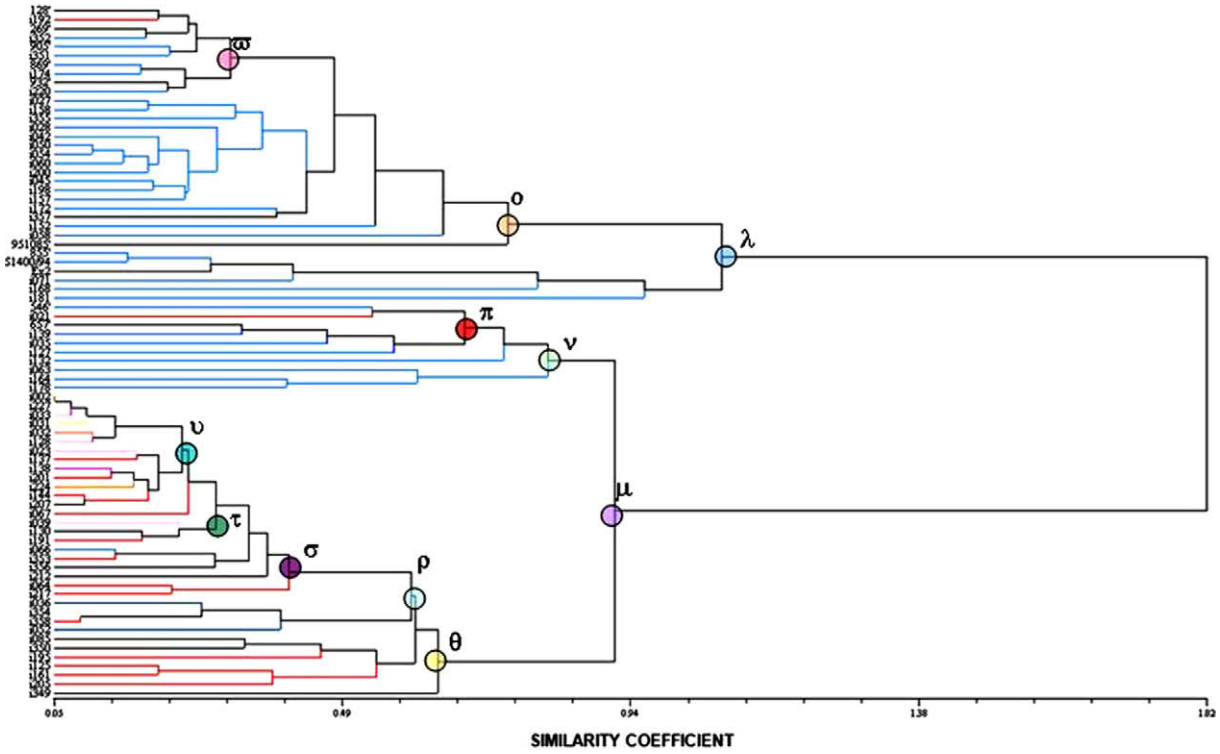

Fig. 6. Dendrogram establishing the relationship between the studied isolates SAHN/UPGMA (quantitative data).

N5. The phage 2/2 was the most infective for serovar Typhimurium strains (47.4%) and for food collected isolates (68.4%). The second (N5) showed the best performance against serovar Enteritidis strains (100%). Both phages were very effective against the clinical isolates (75.6%). In quantitative analysis, investigating the *Salmonella* spp. isolates as OTU's, pointed out the possibility of using the tested phages for phage typing techniques, since it has been possible to separate the two main serovar types into independent branches. There also appears to be a possibility of segregating food and clinical isolates.

In order to create a cocktail of phages effective against several *Salmonella* isolates, a cluster descriptive analysis has been performed, grouping some elements in a simple manner, by association of analyses that have been made. The phages 2/2, N5 and 2/2* forming each a singular group and two more diffuse groups considering the elements 169′, 31M and 12′ to one and the phages 38′, 2δ, 2α, 68′, 135KP, 39′ and 135M to another. Nevertheless, these combinations do not exclude this last choice with one or two more infective phages from the two major groups for the cocktail concept. The Kolmogorov–Smirnov (K–S) was performed with the aim of verifying if each group is an aleatory (random, by chance) sample of a normal, multivariate population. With a result of a *p*-value $\leqslant \alpha$, to a significance value $\alpha = 0.05$, it can be concluded that the distribution of the "phagegroup" variable is not normal (Dallal & Wilkinson, 1986). However, the violations of the normality assumption are not "fatal" and the resultant significance test is still reliable as long as non-normality is caused by skewness and not outliers (Tabachnick & Fidell, 1996). In spite of some deviations to normality being observed, the samples have a significant dimension that allows a discriminant analysis. The results showed that the percentage of correctly classified elements is 92.3%, it being reasonable to distribute the phages into five groups. From an examination of these results the composition of a cocktail (prophylaxis of bacterial infections) can be suggested, containing the bacteriophages: 2/2, N5, 2α, 135KP and 12′. In the EC Project PhageVet-P, one of the *Salmonella* phages studied (phage 38) has been examined for efficacy in controlling gut infections by *S. enteritidis* in 7-day old broiler chicks. Preliminary results indicated that a reduction of 99% in salmonellae recovered from the infected gut contents, could be achieved by orally administering a dose of 10^9 PFU of phage 38 (Gibbs, 2008). Further data will be available in the final report of the EC Project PhageVet-P.

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