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Research Note—

Avian Infectious Bronchitis: Characterization of New Isolates from Italy

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SUMMARY. The isolation of four new variants or serotypes of avian infectious bronchitis virus in Italy is reported. The antigenic characteristics of these strains were investigated by cross-neutralization tests with the new isolates, Fa 6881/97, AZ 27/98, AZ 20/97, and BS 216/01; two of the most common European serotypes, AZ 23/74 and CR 88121 (793B); and the classic Massachusetts M41 serotype in association with a panel of 17 specific antisera. On the basis of the results obtained, the new isolates show relevant serologic differences. In fact, the four isolates were not neutralized by antisera against the most common European and American serotypes; the AZ 20/97 isolate was partially neutralized by FA 6881/97 antiserum but not reciprocally.

The closely related Fa 6881/97 and AZ 27/98 isolates can be considered rather diffused in our country because they have been isolated over 20 times in the last 3 yr in different parts of Italy. On the contrary, the AZ 20/97 and BS 216/01 isolates were reported only once so far.

The reverse transcription–polymerase chain reaction showed that Fa 6881/97 isolate is related to 793B isolate, whereas AZ 27/98 and BS 216/01 isolates appeared not to be related to the most common European and Massachusetts serotypes.

RESUMEN. Nota de Investigación—Bronquitis infecciosa aviar: Caracterización de nuevos aislados de Italia.

Se reporta el aislamiento de cuatro nuevas variantes o serotipos del virus de bronquitis infecciosa en Italia. Las características antiagnéticas de estas cepas se investigaron por medio de pruebas de neutralización cruzada entre los nuevos aislados (Fa 6881/97, AZ 27/98, AZ 20/97 y BS 216/01) con dos de los serotipos Europeos más comunes [AZ 23/74 y CR 88121 (793B)] y el clásico serotipo Massachusetts 41, lo mismo que con un grupo de 17 anticuerpos específicos. Los resultados mostraron que los nuevos aislados mostraron diferencias serológicas relevantes. Cuatro de los aislados no fueron neutralizados por los antisueros contra los serotipos más comunes de Europa y América. El aislado AZ 20/97 fue parcialmente neutralizado por el antisuero contra la cepa FA 6881/97, pero la neutralización no fue recíproca. Los aislados cercanamente relacionados Fa 6881/97 y AZ 27/98 pueden considerarse como ampliamente difundidos en Italia debido a que han sido aislados más de 20 veces durante los últimos 3 años en diferentes partes de Italia. Por el contrario, los aislados AZ 20/97 y BS 216/01 hasta el momento sólo han sido reportados una vez. La prueba de reacción en cadena por la polimerasa-transcriptasa reversa mostró que el aislado Fa 6881/97 está relacionado con el aislado 793B, mientras que los aislados AZ 27/98 y BS 216/01 parecen estar relacionados con los serotipos más comunes Europeos y Massachussets.

Key words: infectious bronchitis virus, serotype, variant, virus neutralization, reverse transcription–polymerase chain reaction

Abbreviations: IBV = infectious bronchitis virus; NI = neutralization index; PCR = polymerase chain reaction; RT = reverse transcription; SPF = specific-pathogen free; VN = virus neutralization
Avian infectious bronchitis can be considered one of the major causes of economic losses to the poultry industry all over the world. The coronavirus, etiologic agent of the disease, first identified in 1936 (1), showed since the mid-1950s (11) the tendency to continuous antigenic changes. Several new infectious bronchitis virus (IBV) serotypes or variants have been reported, particularly in areas of more intensive poultry breeding, above all in the United States and in Europe, but also in many other parts of the world. Actually, they could amount to over 50 (17), but it is believed that just a small proportion of IBVs that exist and are forming have been detected so far (4). The evidence suggested that IBV population seems to be everywhere in a state of continuous flux with appearance of new drifts and shifts in their genome. These last phenomena are typical not only of viruses with segmented RNA, as orthomixovirus and reovirus, but also of viruses with nested RNA, as coronavirus. During the replication of coronavirus, six mRNAs are produced for a mechanism of discontinuous transcription, which can generate new recombinants. Molecular studies have shown that a new serotype can emerge as a result of only a few changes in the amino acid composition in the S1 part of the virus spike protein, with the majority of the virus genome remaining unchanged (5). This could be due to immunologic pressure caused by the widespread use of vaccines, to recombination as consequence of mixed infections, or to the decrease of dominant serotypes as a result of vaccination, allowing other field strains to emerge.

The sharing of so many antigens in the different isolates might suggest that currently available vaccines should be more or less able to provide protection against challenge with viruses belonging to different serotypes from the vaccine itself (9). Furthermore, the tropism of different strains of the virus appears rather variable: respiratory, intestinal, urogenital, mixed. Sometimes an isolate can induce prevalent respiratory symptoms in a flock of one type of chickens and kidney lesions in another one (A. Zanella, pers. obs.). It has also been observed that the tropism of the virus can modify depending on the route of penetration or administration (14).

Our objective is to report on strains of IBV isolated in Italy in these last years, showing different tropism and lesions, and to compare their antigenic characteristics with variants or serotypes already known and diffused in Italy and worldwide.

**MATERIALS AND METHODS**

**Clinical outbreaks.** Fig. 1 is the geographic map indicating the sites and the year of isolation of the four new isolates (Fa 6881/97, AZ 20/97, AZ 27/98, and BS 216/01). Fa 6881/97 was first isolated in a flock of 32-day-old broilers with severe nephritic lesions; AZ 20/97 was from kidneys in a flock of 15-wk-old pullets with nephritis; AZ 27/98 was from a group of 20-wk-old pullets that had only light malaise and respiratory symptoms; BS 216/01 was from the gut in a flock of 23-wk-old layers showing a decrease in egg production and eggshell quality. These four groups of birds originated from different farms; the units were even about 500 km apart from each other and they were not epidemiologically related. All the groups had been vaccinated, once or three times according to the age, with Mass H120 vaccine and, limited to the last group, also with 4/91 vaccine and inactivated vaccine against M41 and Dutch variants.

The Fa 6881/97 and AZ 27/98 isolates can be considered rather diffused in our country because they have been isolated over 20 times in the last 3 yr in different parts of Italy. On the contrary, the AZ 20/97 and BS 216/01 isolates were reported only once so far.

**Substrate.** We used 9- to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (SPA-FAS).

**Virus isolation.** Samples of tracheal exudates and cloacal swabs or kidney tissues, taken, respectively, from chickens suffering from respiratory symptoms or showing severe kidney lesions, were homogenized in phosphate-buffered saline (1:5 w/v), treated with antibiotics, and inoculated in the allantoic sac of 10 embryonated eggs. After incubation at 37 C for 8 days, the eggs were candled daily and examined for specific IBV lesions (17). The allantoic fluids from some eggs were harvested 48–72 hr postinoculation. In order to adapt the virus to the embryo, 10–12 serial passages were performed before the cross-neutralization test.

**IBV strains.** The new isolates, Fa 6881/97, AZ 27/98, AZ 20/97, and BS 216/01, together with two of the most common European serotypes, AZ 23/74 (17) of our collection and CR 88121 (793B) kindly supplied by Dr. J. P. Picault (13), and the reference Mass 41 serotype were included in the study.
Fig. 1. Geographic distribution of the first outbreaks caused by the four new IBV variants isolated in Italy from 1997 to 2001.

**IBV monospecific antisera.** A panel of specific antisera was used: Mass 41, JKM, Arkansas DPI, Gray, Australia T, CR 88121, CR 88221, D274, AZ 23/74, PV 1731/65, AZ 446/66 (17), 793B (reference serum from CLV-Weybridge), and 624I (given by Dr. Capua, Italy). Immune serum was prepared against each of the four new isolates (Fa 6881/97, AZ 27/98, AZ 20/97, and BS 216/01) before the tests were begun. All of the sera were raised in 4- to 6-wk-old SPF chickens, kept in isolation units, inoculated twice at an interval of 21 days, and bled 2 wk later; the sera were filtered by Millipore 0.22 μm, inactivated at 56 C for 30 min, lyophilized, and stored at −20 C (17). The monospecificity of the antisera is well proved by virus neutralization (VN) results.

**VN test.** To establish the possible correlation of new field isolates with some of the most common European, American, and Australian IBV serotypes, VN tests were performed in embryonated eggs, according to the method variant virus–constant serum (log₁₀ virus dilutions and 1:5 serum dilution). Virus and serum were kept in contact for 1 hr and then the mixture was inoculated in eggs (five eggs for dilution). The embryos were candled and examined for specific lesions within 8 days. The neutralization indexes (NIs) were calculated according to the method of Reed and Muench. Only values of NI ≥ 2 log₁₀ were considered positive. In the tests negative serum was included.

**Reverse transcription (RT)–polymerase chain reaction (PCR) test.** RT-PCR was performed according to described methods (6,7,12) on allantoic fluids of embryonated eggs infected with Fa 6881/97, AZ 27/98, AZ 20/97, and BS 216/01 isolates, with primers that can recognize the most common European IBV types (i.e., 793B, D207, B1648, and M41).

RNA was extracted and purified from allantoic fluid by the method of Chomezynski and Sacchi (7), with minor modification, and stored at −20 C. RNA was detected by RT-PCR assay with XCE1+ and XCE2− primers, which are able to amplify a fragment of 464 bp common to all IBV types. Than a nested PCR was performed combining XCE3− with BCE1+, DCE1+, or MCE1+ primers, specific respectively for 793B, D274, and M41 IBV strains.
### RESULTS

**VN test.** The Fa 6881/97 and AZ 27/98 isolates showed a reciprocal cross neutralization (NI > 6.0 log<sub>10</sub>); but they were not neutralized by any other antiserum tested except, weakly, by PV 1731/65 (NI = 2.5). The Fa 6881/97 antiserum showed a weak neutralizing effect on isolates AZ 23/74 and CR 88121 (NI = 2.2 and 2.7, respectively). The reason for such effect is unknown, and whether it is specific or not has not been ascertained. The opposite, in fact, was not evidenced, i.e., antisera to AZ 23/74 and CR 88121 did not neutralize the Fa 6881/97 isolate. As regards the AZ 20/97 isolate, it was partially neutralized only by Fa 6881/97 (NI = 3.5) and PV 1731/65 (NI = 2.8), and, again, there was no reciprocal effect. The AZ 216/01 isolate was not at all neutralized by any considered sera. The complete results are reported in Table 1.

**RT-PCR tests.** The test showed that Fa 6881/97 isolate appears to be related to 793B isolate, unlike the result of the serologic test, whereas AZ 27/98 (antigenically similar to Fa 6881/97) and BS 216/01 isolates appear not related to the most common European serotypes.

### DISCUSSION

The results of reported virologic investigations allowed pointing out the appearance in
Italy of further new variants of IBV, different from the most common serotypes reported in the past and presently, both in Europe and worldwide. The closely related Fa 6881/97 and AZ 27/98 isolates and other similar strains repeatedly isolated in our country were detected at different times and in far-off places of the country from chickens showing respiratory symptoms or kidney lesions. On the basis of numerous isolations (over 20), such a variant or serotype can be considered diffused in our country in the last 3 yr, even though more epidemiologic investigations are needed to better know its real diffusion and persistence.

On the contrary, the AZ 20/97 and BS 216/01 isolates were reported only once. Their results also were different from the previous isolates, even though the first was partially neutralized by Fa 6881/97 antiserum (NI = 3.5).

Preliminary RT-PCR data showed that Fa 6881/97 isolate could be considered related to 793B type, whereas AZ 27/98 and BS 216/01 isolates are not related to the four most common European IBV types (as already shown by VN tests).

In a period of about 30 yr, at least 10 antigenically different isolates have been reported in Italy. Most of them had a transient appearance, nevertheless the clearly nephropathogenic AZ 23/74 serotype has been isolated for over 25 yr (18) and has caused serious damage, particularly in broilers. An efficacious vaccine was also prepared and widely used in the field; after 100 passages in embryo the attenuation appeared very stable (16). Also, the 624I isolate has been present for at least 7 yr but is more localized geographically (2). It is still impossible to foresee what will happen with the new variants. However, stating the present results—the more or less evident neutralizing effect showed by antisera to AZ 27/98 and Fa 6881/97 toward numerous field isolates and the relative high frequency of their isolation—these isolates could become in the future one of the most diffused serotypes in Italy. Further investigations would be necessary, i.e., a more complete molecular characterization by the PCR-restriction fragment length polymorphism method and genome sequencing, for better identifying these new isolates (15), even considering that serotyping and PCR only partially indicate the degree of changes in isolated strains.

A very interesting aspect of IBV epidemiology, as far as it is possible to know, is also the presence and the spreading of the various IBV serotypes in different continents. About 20 emergent serotypes in North America did not spread to other continents; the only exceptions are the Massachusetts and, partially, the Connecticut strains, probably because of their wide use as vaccinal strains. Similarly, the European (over 25), Australian, and Asiatic serotypes apparently did not spread elsewhere. However, research on the matter has been rather poor and it would be worthwhile to conduct more studies. Investigations carried out in Italy on many isolates since the 1960s showed that the majority of them belonged to the Massachusetts serotype. On the contrary, some of them were not neutralized by any antisera from American and Australian serotypes (17). Even in Europe, with exception of 793B (or 4/91 and CR 88121) serotype (8) and the Dutch variants (10), which spread in different countries of the continent (3), certain variants or serotypes were detected for a variable period only in the country or area of origin. Indeed, the spreading of a virus to neighboring areas or countries could be due, at least in part, to its improper introduction by the trading of birds or by the use of attenuated vaccines. In Italy, many isolates obtained from clinically affected chickens induced mortality and specific lesions in embryonated eggs since the first passage (Zanella, pers. obs.). On the contrary, the wild virus requires three to five blind passages before adapting to the embryo and showing typical lesions. Therefore, it is necessary to pay particular attention to such evidence before assuming as natural the appearance in an area or country of new, but sometimes modified, strains.

In conclusion, it is necessary to verify, by a continuous epidemiologic surveillance and by a wide application of improved virologic and serologic methods, if new variants or IBV serotypes are diffused in the same and in different areas or countries and to determine, thereafter, the risk factors and the means of viral transmission.

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